The effect of hydrogen peroxide-producing lactobacilli on wild type and catalase-deficient *Neisseria gonorrhoeae* in a murine model of gonococcal genital tract infection

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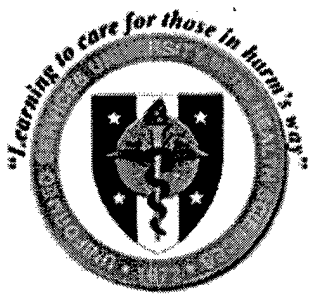

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ABSTRACT

Title of Thesis: The effect of hydrogen peroxide-producing lactobacilli on wild type and catalase-deficient *Neisseria gonorrhoeae* in a murine model of gonococcal genital tract infection

David Jesse Kuch, Master Degree, 2002

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The majority of morbidity associated with *Neisseria gonorrhoeae* (pelvic inflammatory disease, infertility) occurs in women. Survival of the gonococcus in the female genital tract is challenged by a variety of host defenses including inhibitory commensal flora. Lactobacilli, the predominant facultative anaerobic microflora in the female genital tract, produce many potential inhibitory products. We demonstrated the capacity of *L. crispatus* and *L. jensenii*, two human vaginal commensals, to inhibit *N. gonorrhoeae in vitro* and determined the major mediator of inhibition to be hydrogen peroxide. *L. crispatus* was selected as the best candidate to protect against *N. gonorrhoeae* based on greatest *in vitro* inhibition and highest production of lactate. Groups of mice were inoculated intravaginally with *L. crispatus* or left untreated, and challenged with wild-type or catalase mutant FA1090 three hours later. There was no difference in the duration of recovery of either gonococcal strain from mice that were or were not pre-colonized with *L. crispatus*. These data suggest many factors may be involved in the complicated interaction between lactobacilli and gonococci *in vivo*.

THE EFFECT OF HYDROGEN PEROXIDE-PRODUCING LACTOBACILLI ON WILD TYPE AND CATALASE-DEFICIENT *NEISSERIA GONORRHOEAE* IN A MURINE MODEL OF GONOCOCCAL GENITAL TRACT INFECTION

By

Captain David Jesse Kuch

Thesis submitted to the Faculty of the
Molecular and Cellular Biology Program
Uniformed Services University of Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2002

DEDICATION

There are many people throughout the course of my life that have influenced me. I have chosen to dedicate the work described in this thesis to four very important people in my life who have served as an inspiration. First, I am extremely fortunate to have loving and supportive parents who challenged me and allowed me to pursue my dreams. In loving memory of my father, a humble biology teacher, who never understood the impact of his effort. I only wish he could have seen the results of his love and devotion. I will never forget his love of science, unquenchable thirst for knowledge, and unrelenting work ethic. To my mother, who taught me patience, perseverance, and loyalty. To my brother Dan, to whom I share the healthiest of sibling rivalries, which has allowed us to go further in life than we ever expected. Finally and most importantly, I dedicate this work to my loving wife, Sonia. With her at my side, I know that anything is possible. I know the support, patience, and understanding she has displayed during my studies will ensure our love will continue to grow and strengthen.

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Introduction

Overview and Purpose

Due to its clinical importance, the pathogenicity of *Neisseria gonorrhoeae*, the causative agent of the sexual transmitted disease gonorrhea, has been extensively investigated. In 1998, clinicians faced approximately 360,000 cases of gonorrhea, ranking *N. gonorrhoeae* second only to *Chlamydia trachomatis* as the most common cause of infectious disease in the United States (CDC, 1999). The rate of gonorrhea increased in 1998 by 8.9 percent, the first significant increase in almost 14 years. Approximately fifteen percent of men and eighty percent of women infected with *N. gonorrhoeae* are asymptomatic (*Handsfield*, 1990). This high rate of asymptomatic infections contributes to prolonged infections and the spread of infection within communities. Gonorrhea is most detrimental to the health of the female reproductive tract and can result in pelvic inflammatory disease (PID) and sterility via fallopian tube scarring.

Gaining a better understanding of gonococcal pathogenicity may decrease the morbidity caused by this bacterium, and ultimately decrease the costs associated with treatment. Many factors must be evaluated to fully understand the pathogen-host interactions that occur during the different stages of an infection. The female lower genital tract, the primary site of gonococcal infection in women, is a non-sterile environment in which the normal flora define the physiology of the ecosystem. The predominant bacterial inhabitants in this ecosystem are *Lactobacillus spp.* Interestingly, inhibition of *N. gonorrhoeae* by *Lactobacillus species* has been suggested by clinical studies (*Martin et al,* 1999), and directly observed *in vitro* (*Saigh et al*, 1978; *Zheng et al,*

1994). However, the mechanism of the inhibition is not well characterized, and consequently, a source of debate. Potential lactobacillus inhibitory mechanisms include steric hindrance (*Reid et al*, 1993; *Boris et al*, 1998), depletion of growth nutrients, modulation of host immune responses, and the production of antibactericidal compounds such as hydrogen peroxide (*Wheater et al*, 1952), bacterocins (*Barefoot et al*, 1983; *Kanatani et al*, 1995), biosurfactants (*Velraeds et al*, 1998), and/or organic compounds (*Tramer*, 1963). The production of lactate by lactobacilli may inhibit *N. gonorrhoeae* indirectly through acidification of the microenvironment (*Martius et al*, 1988).

In summary, clinical surveys by others suggest that hydrogen peroxide-producing lactobacilli protect females from gonococcal genital tract infection. The inhibitory mechanism utilized by hydrogen peroxide-producing *Lactobacillus spp.* that commonly reside in the human female genital tract (i.e. *L. jensenii* and *L. crispatus*) has not been definitively identified. The central hypothesis of this research endeavor is that *L. jensenii* and *L. crispatus* inhibit *N. gonorrhoeae* through the production of hydrogen peroxide. To test this hypothesis, two main research objectives were established. The first objective was to identify the primary mechanism by which *L. jensenii* and *L. crispatus* inhibit *N. gonorrhoeae in vitro*. The second objective was to evaluate the capacity of these *Lactobacillus spp.* to inhibit *N. gonorrhoeae in vivo* using a murine model of gonococcal genital tract infection as a surrogate model for human infection.

Ecosystem of the Female Lower Urogenital Tract

The female lower genital tract is a complicated ecosystem as a result of numerous factors, many of which are under the control of hormonal fluctuations, environmental conditions (i.e., acidity, oxygen tension), and personal hygiene practices.

Secretions produced by the Bartholin and Skene glands, cervical mucosal cells, and endometrial and fallopian cells are an important component of the vaginal environment (*Larson*, 1993). Although the majority of the secretion composition is water (90-95%), other components are present including inorganic and organic salts, mucin, fatty acids, immunoglobulins, urea, carbohydrates, albumin, iron chelators, lysozymes, and epithelial cell debris. Leukocytes and desquamated epithelial cells are also present in varying concentrations depending on hormonal state.

Both adaptive (specific) and innate (non-specific) immune components help defend the female genital tract from infection. Adaptive immunity is composed of both humoral (antibody) and cellular (T-cell) responses. Vaginal secretions contain both immunoglobulin class G (IgG) and secretory immunoglobulin class A (sIgA) (*Mestecky et al*, 2001). Innate immunity consists of mechanical, cellular, and soluble components that provide a rapid localized response (*Parham*, 2000). Neutrophils, macrophages, and natural killer (NK) cells are the primary cellular innate defenses in the female lower genital tract. The complement system is also an important innate defense against microbial infection (*Parham*, 2000), and is present in vaginal secretions (*Cohen et al*, 1984). Upon activation, complement can directly lyse bacteria, or participate in opsonophagocytosis. Low vaginal pH (acidity), cationic peptides such as defensins and protegrins, and digestive enzymes (i.e., lysozyme) are also involved in the defense of the

lower genital tract. Additionally, the presence of the normal commensal microflora is thought to directly or indirectly afford a protective role against the colonization of pathogens. As a result, normal flora are often considered a component of innate immunity.

A diverse population of commensal microorganisms colonize the exposed body surfaces of healthy individuals. It is approximated that the normal human body contains 10^{14} colony forming units (CFU) of normal flora, composed of 100 different microbial species. The presence of these microbes benefits the host by providing vital physiological functions (*Brooks et al*, 1998). Stimulation of immune responses and protection against pathogens through colonization competition are other beneficial roles of commensal flora. Because of their importance, the absence of normal microbial flora would make life, as we know it, impossible.

From birth, people are continually exposed to a broad range of microorganisms, the result of which is a continual evolution of commensal flora during their life span. Microbes best suited for the "niche" in which they reside proliferate and become the predominant inhabitant. Normal microbial flora are classified as either "residential" (long-term colonization) or "transient" flora (hours to days). Disruption of resident flora leaves the host vulnerable to the colonization and proliferation of pathogens. Normal flora composition is highly complex, and varies depending on site, age, diet, medical treatment (i.e., antibiotics, surgery), anatomic abnormalities, genetic variances, hormonal fluctuations, and environmental conditions. Under rare circumstances (i.e., immune deficiency, immunosuppression), normal flora can produce disease.

The female lower urogenital tract (defined as the anterior urethra, vagina, and endocervix) is a balanced ecosystem of microflora, which is complicated, diverse, and in a state of constant flux (*Levison et al*, 1977). A detailed review of the normal flora found in the female lower genital tract is provided below.

a. ***Commensal Flora of the Female Lower Genital Tract.*** The commensal flora of the female lower genital tract has been studied extensively since 1894, when Döderlein first published a detailed characterization of vaginal normal flora. These early studies revealed the predominant vaginal flora to be Gram-positive rods (originally called Döderlein bacilli), which had the ability to produce and tolerate acid. These bacteria are now classified as *Lactobacillus spp.* Since this definitive study, eighty years of technological advances have improved the characterization of lower genital tract normal flora (*Larson and Falask*, 1980; *Harris et al*, 1928; *Weinstein et al*, 1938; *Hite et al*, 1947). For example, the frequent presence of anaerobic bacteria in vaginal secretions was reported over fifty years ago (*Weinstein et al*, 1938; *Hite et al*, 1947). However, research and clinical communities did not consider anaerobes a major component of vaginal normal flora due to shortcomings in anaerobic specimen collection and identification techniques at the time. Improved anaerobic collection and culturing techniques developed during the 1970s enabled the detection of anaerobic bacteria in the vagina, and thus, generated a more complete description of the female genital tract microflora (*Gorbach et al*, 1973; *Olm et al*, 1975; *Goplerud et al*, 1976). It is now understood that anaerobic bacteria are a major component of the normal genital tract, out-numbering aerobic bacteria 1000 to 1. As the dominant bacterial flora, anaerobes play a

large role in the physiological conditions of the female lower genital tract. For example, anaerobic respiration accounts for the majority of lactic acid in this mucosal site.

The range of bacteria isolated from the genital tract is immense. Brown et al (1989) isolated 4,997 bacterial vaginal isolates from 54 healthy women sampled 9 times over the course of three full menstrual cycles. Of these 4,997 isolates, 40 genera and 94 species were identified and 1,495 isolates were unidentifiable. Although the exact bacterial composition of the lower genital tract varied between studies, the identification of lactobacilli as the predominant genus was unanimous. Healthy pre-menopausal women have 10^7 to 10^8 lactobacilli per gram of vaginal fluid (*Redondo-Lopez et al*, 1990). The most common microbes (both bacterial and fungal) isolated from the genital tract of healthy females as compiled from numerous clinical studies are listed in Table 1.

b. ***Variations in Commensal Flora of the Female Lower Genital Tract.*** The level and composition of normal genital flora are driven by hormonal and environmental factors as well as social factors (e.g., contraceptive type, sexual activity, hygiene practices). Consequently, the normal flora of the female lower genital tract is in a constant state of flux with the capacity to change hourly (*Bruce et al*, 1973).

Hormonal control of genital flora accounts for the microbial variation that is observed as a function of age and menstrual cycle stage. During the birthing process, aerobic lactobacilli obtained from passage through the vaginal canal transiently colonize the infant's vagina. With time, maternal estrogen levels decrease and the female infant enters the pre-menarchal period. This decrease in estrogen correlates with a decrease in lactobacillus colonization and an increase in colonization by a mixed microbial population consisting of cocci and bacilli derived from the skin and colon (*Hill et al*,

Table 1. Most common normal flora of the female lower urogenital tract. [†]

Bacteria	Fungal
Actinomyces spp.	*Candida spp.*
Bacteriodes spp.	
Bifidobacterium spp.	
Clostridium spp.	
Enterococcus spp.	
Enterobacteriaceae	
Eubacterium spp.	
Fusobacterium spp.	
Gardnerella spp.	
Haemophilus spp.	
Lactobacillus spp.	
Mobiluncus spp.	
Mycoplasm spp.	
Peptostreptococcus spp.	
Porphyromonas spp.	
Prevotella spp.	
Propionibacterium spp.	
Staphylococcus spp.	
Streptococcus spp.	
Treponema spp.	
Ureaplasma spp.	

[†]Table based on studies by Goplerlund et al, 1976; Gorbach et al, 1973; Olm et al, 1975; Hiller et al, 1992; Redondo-Lopez et al, 1990; Hill et al, 1995.

1995). At puberty, facultative anaerobic and obligate anaerobic lactobacilli predominate in the lower genital tract. Gram-negative rods, Gram-positive cocci, and yeast also colonize this niche, but to a lesser extent. At menopause, the microbial flora of the genital tract changes again, and is similar to that of the pre-menarchal period unless estrogen therapy is administered. Larsen et al (1982) found that post-menopausal women not receiving estrogen therapy were colonized with lactobacilli less frequently than post-menopausal women receiving estrogen therapy. Another study of post-menopausal women not receiving estrogen therapy found that only forty-nine percent were colonized with lactobacilli, and thirty-eight percent possessed lactobacilli with the ability to produce hydrogen peroxide (*Hillier and Lau,* 1997). These observations suggest that the presence of lactobacilli is under the control of estrogen.

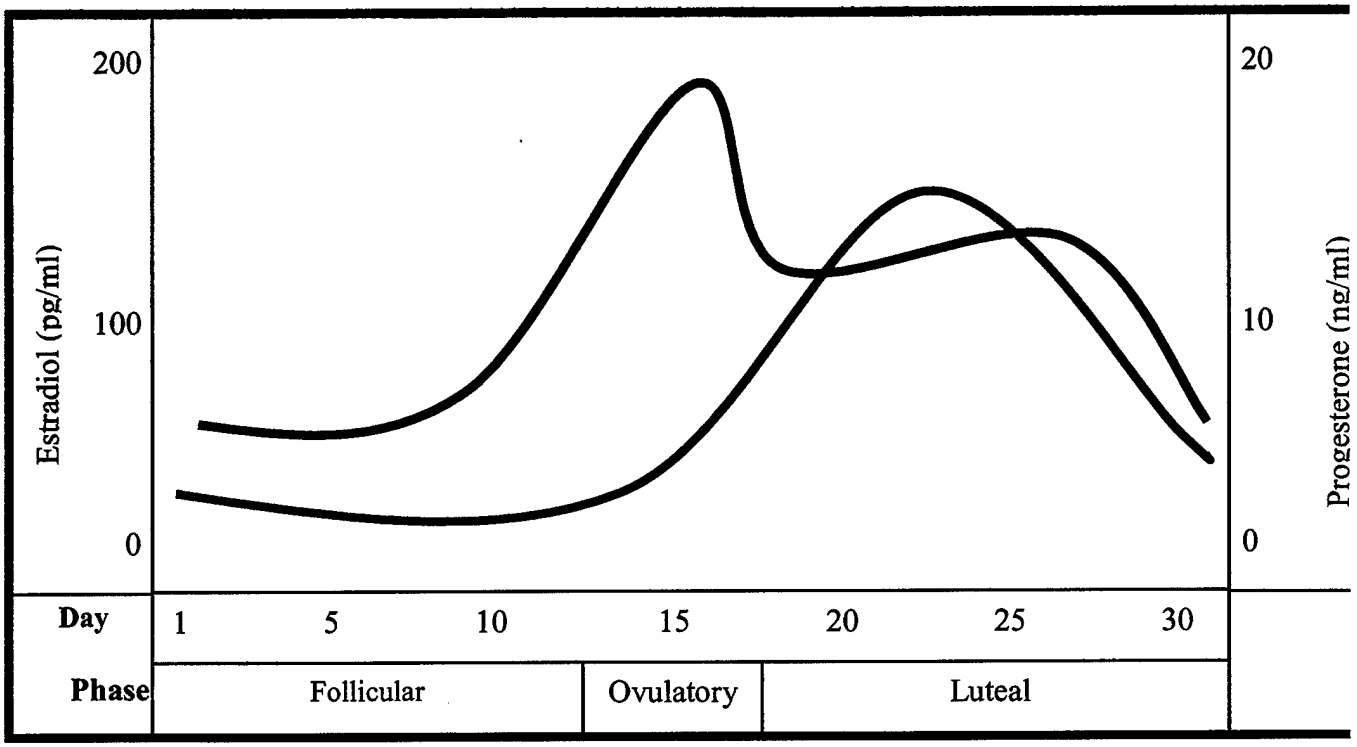
Variation in genital tract normal flora during the menstrual cycle has been extensively studied. Serum levels of estradiol and progesterone over the course of the menstrual cycle are depicted in Figure 1. As shown, a steady rise of estradiol occurs during the first 7 to 8 days of the cycle (follicular phase), which is followed by a sharp increase and subsequent decrease in the middle of the cycle. An estradiol plateau is coupled with a rise in progesterone during the last half of the cycle (luteal phase). Early quantitative surveys revealed that the composition and number of vaginal bacteria varied considerably during the course of the menstrual cycle (*Bartlett et al*, 1977; *Sautter and Brown*, 1980). For example, Bartlett et al (1977) reported that a 100-fold decrease in the average number of bacteria isolated from the vagina is observed during the last week of the menstrual cycle. Others have reported that the follicular phase of the cycle is associated with the highest number of bacteria and lowest number of species (*Brown,*

1982; *Johnson et al*, 1985; Onderdonk et al, 1986; *Wilks and Tabaqchali*, 1987). Those same studies reported a decrease in the total number of bacteria recovered during the luteal phase, coupled with an increase in the variation of microbial species. This trend was attributed to the decrease in lactobacilli, with a subsequent increase in vaginal pH and a resultant instability in microbial colonization (*Brown*, 1982; *Eschenbach et al*, 2000; *Schwebke et al*, 1999).

Direct testing of the effect of estrogen on commensal flora in rats supports the hypothesis that normal flora proliferate under the influence of estrogen. It was observed that ovariectomized rats receiving estrogen had a 100- to 1000-fold increase in bacterial load over untreated ovariectomized rats (*Galask et al*, 1976). This result was consistent with subsequent investigations by Larsen and Galask (1977), and Larsen and Markovetz (1980) that showed the bacterial population density of the rat genital tract was highest during the estrus phase of the cycle, and thus, indirectly related to the concentration of estrogen present.

The composition of vaginal flora is also dependent on social factors. The use of intravaginal tampons as opposed to napkins leads to a decrease in anaerobic bacteria and an increase in coagulase-negative staphylococci (*Chow and Bartlett*, 1989). Increased sexual activity and contraceptive use can also alter the composition of vaginal microflora (Schwebke *et al*, 1999). All of these observations may be attributed to the increase in oxygen tension of the lower genital tract that occurs during sexual intercourse and upon insertion and removal of tampons or a diaphragm. Spermicides containing the active ingredient Nonoxynol-9 may also upset the delicate microbial balance within the lower

Figure 1. Estrogen and progesterone levels with respect to phases of the normal female menstrual cycle.† The blue and red lines represent estrogen and progesterone, respectively. Normal serum estradiol levels peak at 200 pg/ml during ovulation, and then reach a plateau of approximately 100 pg/ml in the luteal phase. Normal serum levels of progesterone are 0.4-0.6 ng/ml in the follicular phase and 10-20 ng/ml in the luteal phase.



† Figure design adapted from Goodman, 1994.

genital tract, based on evidence that lactobacilli are extremely sensitive to this detergent (*McGroarty et al*, 1992).

c. ***Vaginal Acidity***. Vaginal acidity is believed to be an important defense against pathogens in the lower genital tract. The pH of a healthy vagina is acidic (approximately 3.5 to 5.5) due to the metabolism of glycogen to lactic acid (lactate). The vaginal epithelium of females of reproductive age is composed of stratified, non-keratinized squamous epithelial cells. These cells proliferate and shed in response to estrogen. Vaginal glycogen released from desquamated cells is broken down to glucose by vaginal epithelial cells and/or bacteria other than lactobacilli (*Rogosa and Sharpe*, 1960; *Steward-Tule,* 1964). Once converted, vaginal glucose is then anaerobically metabolized by lactobacilli to lactic acid, the primary molecule responsible for vaginal acidification. The acidity generated by lactobacilli provides a favorable and selective environment for acid tolerant microorganisms such as *Lactobacillus spp*. It is well understood that the increased presence of glycogen in vaginal epithelial tissue is directly under estrogen control (*Cruickshank and Sharmon*, 1934; *Weinstein and Howard*, 1939). Consistent with hormonal control of glycogen production and vaginal normal flora, the pH of the vagina is lowest in the luteal phase of the menstrual cycle.

Although the physiology behind vaginal pH is well understood, the source of vaginal acidity has been long debated. However, although both vaginal epithelial cells and bacteria can convert glucose to lactate, there is a great deal of evidence that the majority of vaginal lactate is bacterial in origin. Doderlein (1892) first postulated that vaginal lactobacilli produced the majority of vaginal acid, and therefore, were responsible for the low pH and innate resistance of the vagina to pathogens. Support of this

hypothesis was provided through clinical studies by Miura (1928; summarized in *Weinstein and Howard*, 1939) and Cruickshank (1934), which found that lactobacilli and vaginal glycogen were required for vaginal acidity. *In vitro* studies by Wylie (1969) demonstrated the ability of *L. acidophilis* to acidify human female genital tract secretions. Consistent with the conclusion that bacteria are the major source of acidity, Boskey et al (1999) found that acidophilic lactobacilli create an acidic environment. Decreased media pH was directly proportional to bacterial CFU. Acid production rates of *Lactobacillus spp.* commonly isolated from the female lower genital tract were approximately 10^6 protons per second. Once environmental acidity was achieved (pH 3.2 to 4.8), the bacterial growth rate decreased and acid production increased. The authors also estimated 10^8 CFU/vagina to be the minimum number of lactobacilli required to achieve acidic conditions in the healthy vagina.

A direct correlation between the amount of lactobacilli present and vaginal acidity was observed in a quantitative clinical survey of vaginal microflora in which the vagina was found to be most acidic when the density of lactobacilli was highest (*Sautter and Brown*, 1980). Clinical studies on the lower genital tract of pre-pubescent females revealed the presence of fewer than 10^5 lactobacilli/gram (approximately equivalent to 10^5 lactobacilli/mL) of vaginal fluid (*Hill et al*, 1995). The low numbers of vaginal lactobacilli coupled with low vaginal glycogen levels results in a neutral vaginal pH in these females. Conversely, healthy women of reproductive age were found to possess 10^7 to 10^8 lactobacilli/mL of vaginal fluid (*Onderdonk et al*, 1977; *Redondo-Lopez et al*, 1990). The presence of high levels of lactobacilli combined with higher levels of glycogen (due to elevated estrogen levels following puberty) results in optimal vaginal

acidity. In females of reproductive age, the absence of lactobacilli and the resultant neutral vaginal pH is considered pathological due to the overgrowth of other anaerobic bacteria (e.g., *Gardnerella vaginalis, Prevotella bivia, Peptostreptococcus anaerobius*). This condition is referred to as bacterial vaginosis.

Technological advances and improved laboratory analysis have provided definitive evidence for Doderlein's original hypothesis. Definitive physiological proof that bacteria are primarily responsible for vaginal acidification comes from studies that used the chirality of lactate to differentiate between prokaryote- and eukaryote-generated lactate. Based on relative amounts of D-lactate and L-lactate enantiomers in vaginal secretions, it was calculated that 80 percent of vaginal acidity is derived from the anaerobic metabolism of glucose by lactobacilli, while the remaining 20 percent is produced by vaginal epithelial cells (*Boskey et al*, 2001).

As discussed previously, the microbial inhabitants of the lower female genital tract fluctuate as a function of age. This same trend has been observed for vaginal pH. During the initial couple of weeks following birth, the vaginal pH is acidic due to the presence of both lactobacilli and glycogen, which are maternal in origin and reside temporarily in the infant (*Mandar and Mikelsar,* 1996). Following the initial but brief acidic phase after birth, the prepubescent vagina contains minimal glycogen and glucose; a change in the microbial population coupled with an absence of glycogen results in a more neutral vaginal pH. At puberty, glycogen, under the control of estrogen, is deposited in vaginal epithelial tissue. Host eukaryotic cell enzymes convert the glycogen to the more usable form of glucose. Glucose is then metabolized to lactate by lactobacilli present in the genital tract and vaginal acidity results.

Clinical studies on females receiving estrogen therapy have provided further insight into the complex relationship between lactobacilli, glycogen, and vaginal acidity. Research conducted by two independent laboratories demonstrated a decrease in vaginal pH following estrogen therapy, however, there was no difference in vaginal pH among women that were colonized by lactobacilli or not (*Larson and Galask*, 1980; *Ginkel et al*, 1993). These observations suggest both vaginal lactobacilli and glycogen are required to attain an acidic vaginal pH.

Lactobacillus spp. as a Host Innate Defense

Lactobacillus spp. are Gram-positive, non-spore-forming bacilli that commonly colonize the human oral, gastrointestinal, and female genital tracts. Lactobacilli are rarely associated with serious human infections, and medical importance is limited to opportunistic infections in immunocompromised patients. These facultative anaerobic bacilli are described as aerotolerant anaerobic bacteria that ferment glucose to lactic acid (lactate). Only four percent of vaginal lactobacilli are classified as strict anaerobes (*Eschenbach et al*, 1989). Consequently, lactobacilli are categorized with lactic acid bacteria. Other genera in this group are *Streptococcus, Sporolactobacillus, Pediococcus, Bifidobacterium,* and *Leuconostoc.* As a group, lactic acid bacteria share unusual metabolic and nutritional properties. These bacteria generate ATP via the fermentation of carbohydrates and form lactic acid as a major end product. This ability to produce and tolerate high levels of acidity provides a competitive edge to the bacteria through the exclusion of acidophobic microorganisms. Additionally, in general all lactic acid bacteria are facultative anaerobes that do not contain cytochromes (*Stanier et al*, 1976).

Knowledge of lactobacillus biology has been useful in the agricultural, dietary supplementation, and culinary industries. Depending on environmental conditions, lactobacilli can secrete many by-products into their surrounding environment. Many of those by-products can be inhibitory to surrounding bacteria (Figure 2).

a. *Metabolism*. Lactic acid bacteria utilize glucose to generate adenosine triphosphate (ATP) through the anaerobic processes of homofermentation or heterofermentation. A majority of *Lactobacillus spp.* utilize homolactic fermentation as shown in Figure 3. This form of fermentation generates ATP by means of the Embden-Meyerhof-Parnas (EMP) pathway. One molecule of glucose is biochemically converted into two molecules of pyruvate to derive a net gain of two molecules of ATP. Lactate dehydrogenase then catalyzes the reduction of pyruvate to lactate, oxidizing two molecules of NAD, which were previously reduced during the EMP pathway. The lactic acid (lactate) is then excreted by the bacterium, helping to maintain an acidic environmental pH in which lactobacilli flourish. Heterofermentation produces a mixture of products including formate, acetate, succinate, lactate and ethanol. The exact composition of products is dependent on the conditions in which the fermentation occurred. Some homofermentative organisms produce a wide range of products under altered environmental conditions. Because fermentation is an anaerobic process, oxygen is not utilized as a final electron acceptor. In other words, oxidative fermentation does not occur (*Moat and Foster*, 1995; *Neidhardt et al*, 1990).

Figure 2. Potential inhibitory mechanisms of lactobacilli. Figure adapted from *Reid*, 2001.

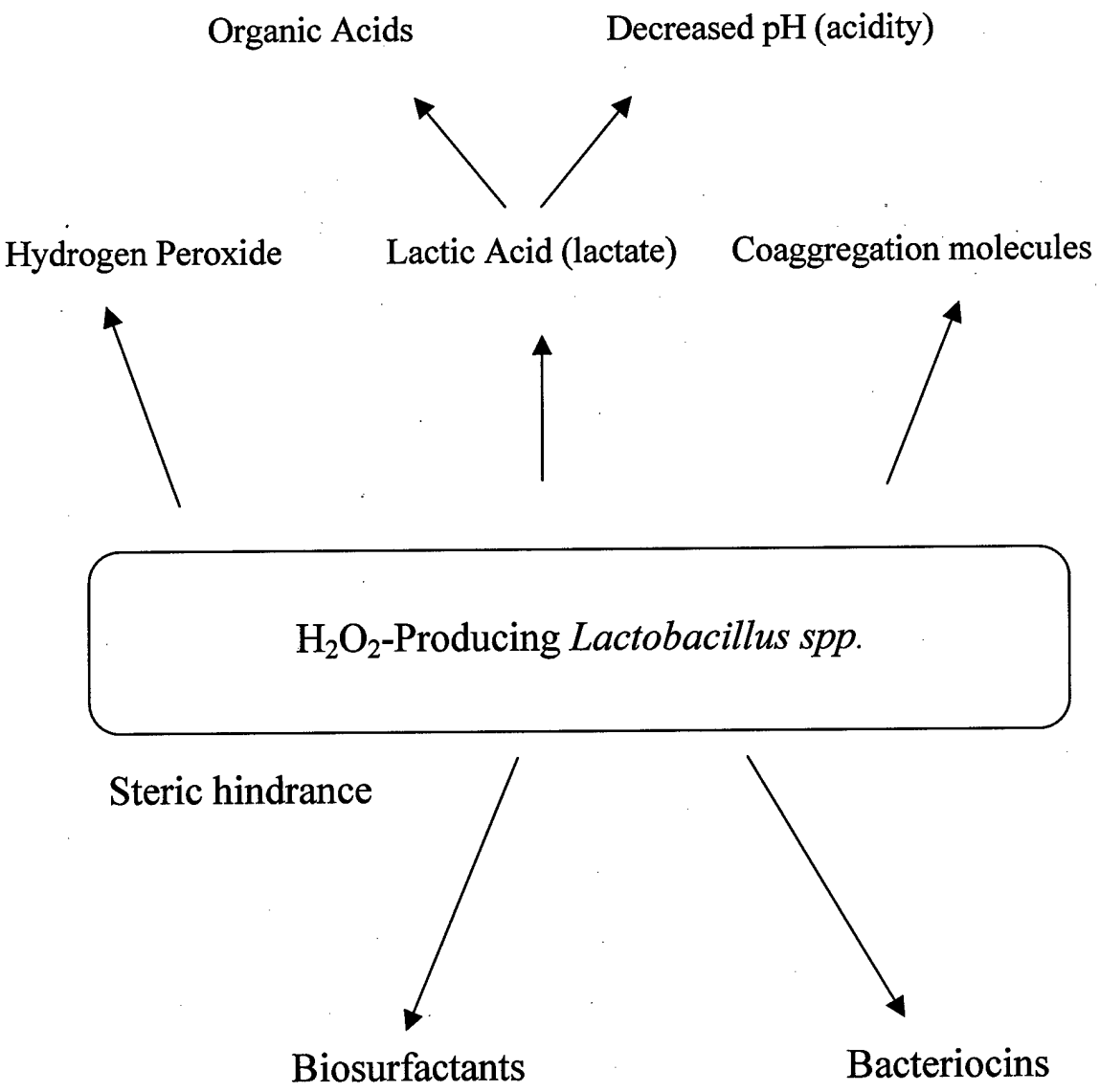
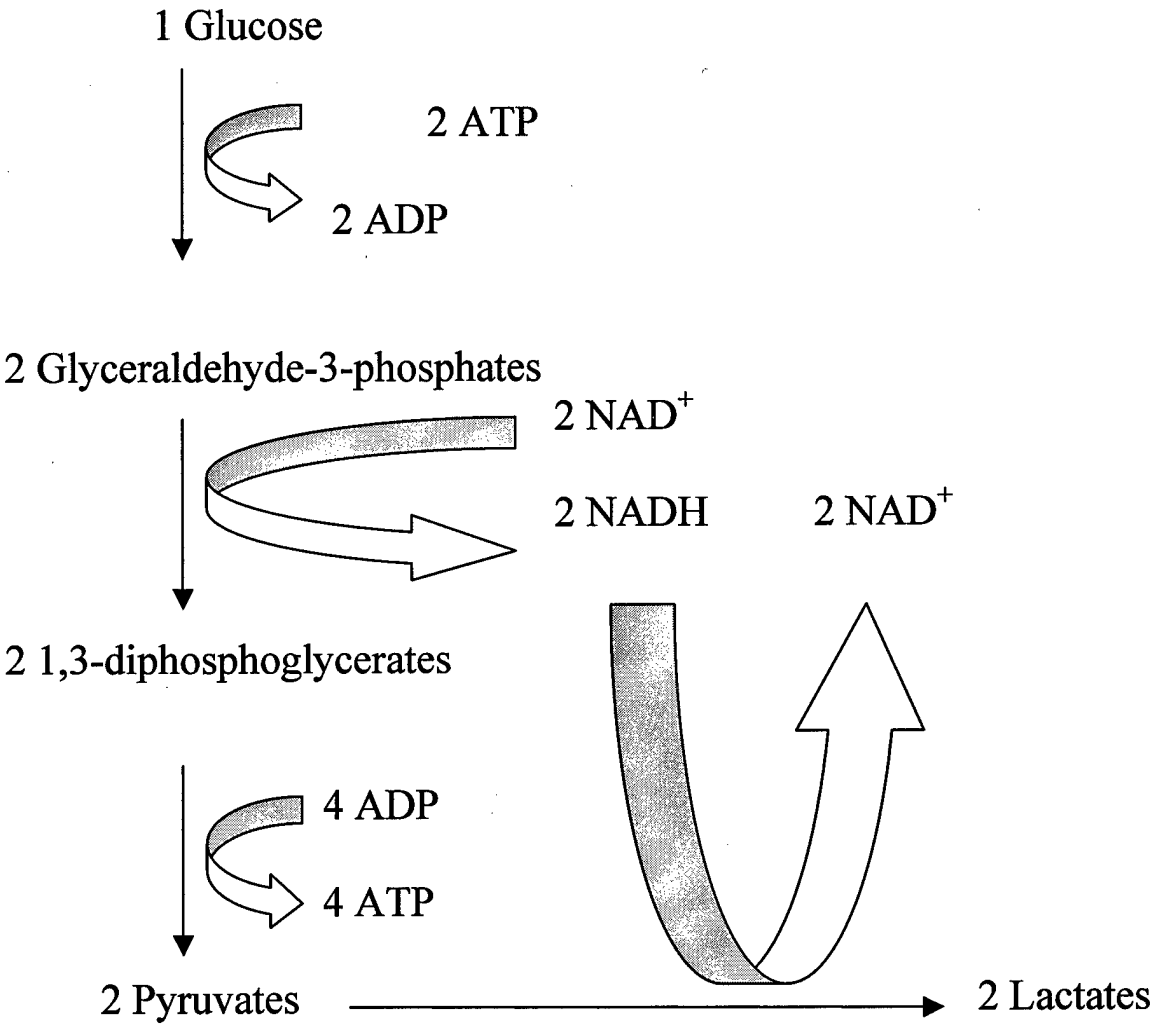


Figure 3. Homolactic fermentation utilized by *Lactobacillus spp.* Figure adapted from *Neidhardt et al*, 1990.



As mentioned previously, lactic acid produced by lactobacilli is the major contributor to vaginal acidity. Although vaginal acidity itself can inhibit non-acidophilic bacteria, organic compounds (i.e., acetic acid, lactic acid) can also be directly inhibitory to bacteria. Early studies on the inhibition of *Staphylococcus spp.* and *E. coli* by *L. acidophilus* concluded that lactic acid was strongly bactericidal (*Tramer*, 1966). More recent *in vitro* assays showed that lactate produced by *L. crispatus* inhibited *Salmonella enterica servar Enteritidis* at a pH of 5.8 (*van der Wielen et al*, 2002).

b. ***Generation of Hydrogen Peroxide.*** Hydrogen peroxide, an oxidizing agent in most chemical reactions, is an anti-bacterial compound commonly associated with the respiratory burst of phagocytic immune cells. Hydrogen peroxide, however, can also be produced by some bacterial strains as a by-product of metabolism. This bacterial by-product can potentially serve as a defense mechanism under the right circumstances. It is well documented that hydrogen peroxide produced by one bacterial species can inhibit the growth of another species (*Dahiya and Speck*, 1968; *Thompson and Johnson*, 1950, *Wheater et al*, 1952). Hydrogen peroxide can diffuse across cellular membranes and disrupt molecular (DNA damage, protein oxidation, lipid peroxidation) and cellular (signal transduction, membrane transport, gene expression, cellular membrane function) processes within the bacterium (*Weiss*, 1986).

As described, most lactobacilli are facultative anaerobes, and consequently, do not require molecular oxygen to generate ATP via fermentation. However, in the presence of oxygen, lactobacilli can use oxygen as an alternative electron acceptor and generate reactive oxygen species, most notably superoxide and hydrogen peroxide. Lactobacilli, as well as other lactic acid bacteria, do not possess heme, and therefore,

cannot utilize the cytochrome system to reduce oxygen to water during terminal oxidation (*Eschenbach*, 1989). In the absence of heme, lactobacilli use flavoproteins, which convert molecular oxygen to hydrogen peroxide (*Whittenbury*, 1964). The generation of these reactive oxygen species is described by the biochemical reaction: L-Lactate + O_2 + $FADH_2$ → Pyruvate + H_2O_2 + FAD (*Moat and Foster*, 1995; *Neidhardt et al*, 1990; *Lengeler, et al*, 1999). The enzyme lactate oxidase catalyzes the conversion of lactate in the presence of oxygen to pyruvate and hydrogen peroxide. During the reaction, oxygen serves as a hydrogen acceptor/electron sink for the auto-oxidation of flavin adenine dinucleotide (FAD), a flavoprotein within the cytoplasm of the lactic acid bacterium. FAD is required for the reaction, and serves as an electron carrier (co-enzyme). No energy is conserved over the course of the reaction.

In the absence of catalase, this reaction results in the formation of excessive amounts of hydrogen peroxide that cannot be degraded by the lactobacilli, and thus must be excreted to avoid toxicity. Excreted hydrogen peroxide can inhibit or kill bacteria that do not possess hydrogen peroxide-scavenging enzymes such as catalase and peroxidases. Consequently, it is theorized that hydrogen peroxide-producing lactobacilli may defend mucosal surfaces such as the vagina and/or endocervix. A compilation of data from several studies suggests that the lower genital tract of 42 to 74% of healthy females between the ages of 16 and 45 years are colonized with hydrogen peroxide-producing lactobacilli (*Hillier et al*, 1992 and 1993; *Hawes et al*, 1996; *Puapermopoonsiri et al*, 1996). Despite minor variations in the reported prevalence of these microbes among women, lactobacilli were the predominate bacteria in these studies.

The hydrogen peroxide-producing ability of different lactobacillus strains has been quantified *in vitro*. Wheater et al (1952) observed that hydrogen peroxide was produced by *L. lactis* in the presence of trace levels of oxygen; the levels of hydrogen peroxide produced were sufficient to inhibit *S. aureus*. Another *in vitro* study revealed that hydrogen peroxide was present when oxygen was supplied through culture agitation (*Ocana et al*, 1999a). Of importance to our study, greater than 94% of clinical isolates of *L. crispatus* and *L. jensenii,* the two most prevelant *Lactobacillus spp.* in the female genital tract, produced hydrogen peroxide *in vitro* (*Antonio et al*, 1999).

c. Predominant Lactobacillus spp. of the Female Lower Genital Tract. Historically, the identification of lactobacilli at the species level has been unreliable due to shortcomings of specimen collection and laboratory identification techniques. The use of sugar fermentation and other phenotypic assays (*Rogosa*, 1960) served as the benchmark until the advent of DNA homology-based identification techniques. Consequently, lactobacilli were identified only at the genus level in early studies, and major taxonomic changes within the genus have since occurred. The most significant taxonomic change is the division of the *L. acidophilus* group into two distinct groups, containing 6 different species that were not distinguishable by original conventional biochemical assays (*Johnson et al*, 1980).

The development of more rapid and precise DNA-based identification techniques has further improved the identification of *Lactobacillus species*. For example, the technique of random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) was employed to generate lactic dehydrogenase (LDH) electrophoretic gene profiles in the differentiation of *Lactobacillus spp.* (*DuPlessis and Dicks*, 1995).

Restriction analysis of the amplified 16S-ribosomal region of lactobacillus DNA, a technique called rapid amplified ribosomal DNA restriction analysis (ARDRA) is another reliable and rapid method to identify *Lactobacillus species* at the species-level (*Ventura et al,* 2000). More recently, multiplex PCR assays, which incorporate primers designed from the 16S-23S ribosomal RNA intergenic spacer region of lactobacilli were utilized to speciate lactobacilli (*Song et al*, 2000).

These improved identification techniques have led to the isolation and characterization of approximately 134 different species of lactobacillus from the human vagina (*Ocana et al*, 1999a). Giorgi et al (1987) reported that *L. gasseri*, *L. fermentum*, *L. cellobiosus, L. crispatus,* and *L. jensenii* were the predominant vaginal *Lactobacillus spp.* A similar study reported that *L. crispatus* and *L. jensenii* were the predominant genital tract lactobacilli with isolation rates of 32% and 23%, respectively (*Antonio et al*, 1999). Interestingly, *L. acidophilus*, which was previously considered to be the predominant *Lactobacillus spp.* in the genital tract, was not recovered in either study conducted by *Antonio et al* (1999) and *Giorgi et al* (1987).

Due to the large number of lactobacilli in the female lower genital tract and the dynamic nature of this environment, it is important to classify vaginal lactobacilli as either persistent or transient colonizers. An eight-month clinical study revealed hydrogen peroxide-producing strains of *L. crispatus* and *L. jensenii* were persistent colonizers of the vagina. Conversely, *Lactobacillus spp.* other than *L. crispatus* or *L. jensenii,* and non-hydrogen peroxide-producing lactobacilli only transiently colonized the vagina (*Vallor et al*, 2001). These observations suggest production of hydrogen peroxide by lactobacilli may enhance the ability to sustain long-term vaginal colonization.

d. ***Lactobacillus spp. as a probiotic.*** The term bacteriotherapy, or probiotics, is defined as "a viable mono or mixed culture of microorganisms, which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (*Havenaar et al*, 1992). The central premise of this therapeutic application is that most microbes produce by-products and/or conditions (e.g., acidity) that inhibit other microbes. Louis Pasteur, working with Joubert, first postulated the therapeutic application of bacteria when they observed the ability of "common bacteria" to inhibit the *in vitro* and *in vivo* growth of anthrax bacilli (translated by *Jack et al*, 1995 from C.R. Soc. Biology Paris 85:101-115). A major advantage of this form of therapy is to provide an alternative treatment for infectious disease that will ultimately reduce the rising incidence of antibiotic resistance. Additionally, treatment costs associated with infection would be drastically reduced. However, the use of probiotics is not without disadvantages, as even "benign" probiotic microorganisms can become opportunistic if the patient is immunocompomised, or immunosuppressed (*Harty et al*, 1994; *Rautio et al*, 1999).

Lactobacillus species have been used to prevent the colonization of pathogens in the human gastrointestinal (*Isolauri et al*, 1991; *Tannock et al*, 2000; *Ogawa et al*, 2001), urinary (*Reid et al*, 2001; *Gupta et al*, 1998), oral (*Sookkhee et al*, 2001), and genital tracts (*Eschenbach*, 1989; *Hillier et al*, 1992; *Pybus and Onderdonk*, 1999; *Ocana et al*, 1999a; *McLean and Rosenstein*, 2000; *Famularo et al*, 2001). Recently, lactobacillus-mediated anti-tumor activity was also described (*Bauer*, 2001). In theory, lactobacilli could serve as a vaginal probiotic, and/or prophylactic, which would provide a natural, long-term barrier against pathogens. It is well documented that the presence of high

numbers of lactobacilli in the genital tract prevents the overgrowth of bacteria associated with bacterial vaginosis (*Eschenbach,* 1989; *Hillier et al*, 1992; *Pybus and Onderdonk*, 1999; *McLean and Rosenstein*, 2000; *Famularo et al*, 2001). A clinical survey conducted by Hillier et al (1992) revealed women colonized with hydrogen peroxide-producing lactobacilli had a reduced prevalence of vaginal candidiasis, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and viridians streptococci. *In vitro, L. paracasei* inhibited *Staphylococcus aureus* (*Ocana et al*, 2001).

Since lactobacilli are considered the major protective vaginal flora, a probiotic innovation to restore lactobacilli in females that lack this flora is being actively sought. In the search for the best possible vaginal probiotic strain of lactobacilli, Ocana et al (1999) screened 35 hydrogen peroxide-producing lactobacillus strains. After assessing hydrogen peroxide production ability and tissue adherence, *L. crispatus* strain F117, which produced the highest level of hydrogen peroxide, was found to have the most potential as a vaginal probiotic. Once selected, the inhibitory capacity of *L. crispatus* strain F117 was tested on the uropathogen *S. aureus*. Inhibition of *S. aureus* was observed to be a function of oxygen availability and the initial concentration of *L. crispatus*. Vallor et al (2001) evaluated the ability of lactobacilli to colonize the human vagina, and found that *L. crispatus* and *L. jensenii* were persistent colonizers. These species were, therefore, considered to have the best potential for probiotic utilization.

The route by which a probiotic lactobacillus product could most effectively be delivered has also been investigated. Delivery systems have ranged from the use of dairy products containing lactobacilli (i.e., yogurt, milk) to lactobacillus vaginal suppositories (*Hughes and Hillier*, 1990). Many of these products are still under development, and/or

are undergoing phase II clinical trials. Paraje et al (2000) tested the viability of lactobacillus-containing tablets using different pharmaceutical compositions. This study found polyethyleneglycol tablets to be the best preparation, in that they retain the capacity to produce vaginal lactobacillus levels between 10^6 to 10^7 CFU for up to one year following tablet insertion. Reid et al (2001) showed that vaginal administration of probiotic strains of lactobacilli increased the long-term concentration of vaginal lactobacilli. Another, unrelated clinical study concluded that orally delivered lactobacilli via a milk-suspension restored vaginal lactobacilli and prevented recurrent yeast vaginitis, bacterial vaginosis, and urinary tract infections (*Reid et al*, 2001). Finally, successful treatment of bacterial vaginosis (BV) through the use of a vaginal tablet containing a minimum of 10^7 CFU of hydrogen peroxide-producing *L. acidophilus* and 0.03 mg of estradiol was also reported (*Parent et al*, 1996).

Neisseria gonorrhoeae Defenses Against Inhibitory Normal Flora

Neisseria gonorrhoeae is a persistent human-specific urogenital pathogen that most commonly colonizes the lower urogential tract. During a localized gonococcal infection, *N. gonorrhoeae* adheres to, and invades epithelial cells of the lower genital tract. Due to the non-sterile condition of this niche in females, *N. gonorrhoeae* must interact with normal flora during infection of women.

The observation that some patients appeared to be resistant to gonococcal infection prompted investigation of natural protective mechanisms against gonorrhea. Kraus and Ellison (1974) isolated aerobic urethral flora from men who failed to develop gonorrhea after exposure. *Staphylococcus epidermidis* was consistently cultured from the

urethra of these men, and the *S. epidermidis* strains inhibited *N. gonorrhoeae in vitro*. This early study generated the hypothesis that bacterial interference could confer protection against gonorrhea. A subsequent study revealed that *N. meningitidis*, *S. epidermidis*, *Corynebacterium species*, *Staphylococcus aureus*, Group A streptococcus, and *N. subflava* inhibited the growth of *N. gonorrhoeae in vitro*, while Group B streptococcus, *Streptococcus viridians*, *Lactobacillus sp. Acinetobacter calcoaceticus*, and *Escherichia coli* failed to inhibit *N. gonorrhoeae* (*Shtibel*, 1976). In a screen of vaginal normal flora isolates, Kaye and Levison (1977) reported that most strains of *S. epidermidis* and *S. aureus* inhibited *N. gonorrhoeae in vitro*. Conversely, only some strains of *S. viridians*, *Neisseria spp.*, *Candida spp.*, and *Bifidobacterium* inhibited gonococcal growth. In contrast to the studies above, *McBride et al*, (1978) did not identify any particular cervical bacterial composition from women that was conducive or inhibitory to gonococci *in vitro*. The authors concluded that resistance to gonorrhea was the result of multiple factors including physiological and immunological factors.

Saigh et al (1978) conducted the first major *in vitro* study that demonstrated inhibition of *N. gonorrhoeae* by lactobacilli. This research team demonstrated that endocervical isolates of streptococci, staphylococci, and lactobacilli (in order of inhibition ability) significantly inhibited growth of *N. gonorrhoeae* in a gel overlay assay. The exact mechanism of this inhibition, however, was not identified.

Saigh et al (1978) also provided *in vivo* evidence in support of their *in vitro* observations described above. High concentrations of inhibitory lactobacilli were associated with a decreased incidence of gonorrhea in women who were exposed to *N. gonorrhoeae*. The concentration of lactobacilli isolated from the endocervix was the only

significant difference in microbial flora between infected and uninfected women. Consistent with the hypothesis that lactobacilli protect against *N. gonorrhoeae*, the authors also observed an increased incidence of gonorrhea the week preceding menses, when concentrations of lactobacilli are lowest.

Subsequent clinical studies have supported Saigh's original hypothesis that the risk of acquiring gonorrhea is directly related to the absence or decreased presence of lactobacilli (*Hillier et al*, 1992; *Martin et al*, 1999). Although some reports attribute this protection to hydrogen peroxide (*Hillier et al*, 1992), a clinical survey by Martin et al (1999) showed that both hydrogen peroxide-producing and non-hydrogen peroxide-producing lactobacilli in the vaginal tract were equally protective against gonorrhea, a result that suggests another lactobacillus factor is responsible for the inhibition.

Theoretically, gonococcal catalase should protect against hydrogen peroxide-producing lactobacillus. Catalase is an antioxidant protein that catalytically converts hydrogen peroxide to water and molecular oxygen. The detoxification of hydrogen peroxide prevents the damage to DNA (i.e., single-strand breaks, chemical alteration of thymidine), which could result in mutations or cell death (*Ananthaswamy and Eisenstark*, 1977; *Demple and Linn*, 1982). Consequently, catalase maintains both the cellular and molecular integrity of the gonococcus. The enzymatic activity of gonococcal catalase is limited to the cytoplasm (*Zheng et al*, 1992). This theory that gonococcal catalase protects against hydrogen peroxide is supported by the demonstration that catalase-deficient gonococcal mutants were more sensitive to hydrogen peroxide *in vivo* than gonococci possessing catalase activity (*Johnson et al*, 1993; *Soler-Garcia*, 2002).

The effect of *in vitro* culture conditions on gonococcal catalase production were studied by Zheng et al (1994) to elucidate why gonococcal catalase does or does not protect against hydrogen peroxide-producing lactobacillus. At neutral pH, hydrogen peroxide-producing *L. acidophilus* inhibited a catalase-deficient strain of *N. gonorrhoeae*, but failed to inhibit wild-type gonococci. Increased catalase activity was detected at neutral pH; this result suggests gonococcal catalase is induced by lactobacilli. Wild-type *N. gonorrhoeae* was inhibited by *L. acidophilus* at acidic culture conditions. The observed inhibition was neutralized by the addition of bovine catalase. At low pH, gonococcal catalase activity was decreased when *N. gonorrhoeae* was co-cultured with *L. acidophilus*. Analysis of spent growth media from the lactobacilli suggested *L. acidophilus* produces a heat-stable protein during acidic conditions that directly inhibits gonococcal catalase production and/or activity. The group concluded that a multitude of factors (i.e., hydrogen peroxide, bacteriocins, acidity) play a role in inhibition of gonococci. Consistent with these results is the identification a bacteriocin produced by vaginal *L. salivaris* subspecies *salivarius* (*Ocana et al*, 1999a). *In vitro* analysis showed this protein had an inhibitory effect on *N. gonorrhoeae*.

MATERIALS AND METHODS

I. Bacterial Strains and Culture Conditions. *L. murinus* strain LB10A, a mouse vaginal isolate (*Jerse et al*, 2002) and streptomycin-resistant derivatives of *L. crispatus* (ATCC 33197) and *L. jensenii* (ATCC 25258), which are human urine and vaginal isolates respectfully, were utilized in these studies. Both human isolates produced hydrogen peroxide as detected by the qualitative assay described below; strain LB10A did not produce detectible levels of hydrogen peroxide by this assay. All strains were resistant to trimethoprim at concentrations greater than 400 μg/ml. *L. murinus* was resistant to streptomycin, but the two human isolates were sensitive. The minimum inhibitory concentration (MIC) for streptomycin against the *L. crispatus* and *L. jensenii* strains was determined to be 25 μg/ml by standard methods. Streptomycin resistant isolates of both *L. crispatus* and *L. jensenii* were isolated for use in both *in vitro* and *in vivo* experiments by serial passage in increasing concentrations of streptomycin. Although other spontaneous mutations could have occurred through the utilization of this method, the streptomycin resistant *L. crispatus* and *L. jensenii* strains possessed the same capability to inhibit *N. gonorrhoeae in vitro* as the parent strains. *N. gonorrhoeae* FA1090 (wild-type) (*Cohen et al*, 1994) and 24.2, a genetically defined catalase-deficient mutant of FA1090 (*Soler-Garcia*, 2002), were used for both *in vitro* and *in vivo* experimentation. Gonococcal strain FA1090 is a serum resistant strain isolated from a female with disseminated gonococcal infection. Gonococcal strain 24.2 carries a 1.2-kilobase (kb) internal deletion in the catalase gene (*kat*).

Culture conditions, unless otherwise stated, were as follows. Lactobacillus strains were cultured on Lactobacillus Mann-Rogosa-Sharpe (L-MRS) agar and in L-MRS broth.

All cultures were incubated at 37°C in a humid atmosphere containing 5% carbon dioxide. Gonococci were cultured on GC agar with 5 grams/liter Bacto agar containing Kellogg's supplements (*Jerse et al*, 1994). Incubation conditions for *N. gonorrhoeae* were 37°C in a humid atmosphere containing 7% carbon dioxide. All culture media used throughout the described research were purchased from Difco Laboratories (Detroit, MI).

II. Gel Overlay Assays. A modification of the gel overlay assay described by Saigh et al (1978) was used to study the *in vitro* inhibition of *N. gonorrhoeae* by hydrogen peroxide-producing lactobacilli. A suspension of each lactobacillus strain tested was prepared by suspending lactobacilli harvested from L-MRS agar in sterile saline to an initial optical density at 600 nm (OD_{600}) of 0.30. Serial dilutions of the bacterial suspension were cultured to determine the concentration of viable lactobacilli via standard protocol. The concentration of the undiluted lactobacilli varied between strains: *L. crispatus* was 3.6 x 10^6 CFU/ml, *L. jensenii* was 3.8 x 10^7 CFU/ml, and *L. murinus* LB10A was 2 x 10^7 CFU/ml. Fifty-µl of the undiluted and 10^{-1}, 10^{-2}, and 10^{-3} diluted suspensions were used to create "spot inoculations" on heart infusion agar (HIA) at pH 5.8 and 7.2. Media pH was adjusted prior to autoclaving with 1N hydrochloric acid or 1N sodium hydroxide respectively. Once "spot inoculations" of lactobacilli were dry (approximately 30 minutes at room temperature), the plates were incubated at 37°C in 5% carbon dioxide. Following 20-24 hours of incubation, approximately 8 ml of supplemented GC agar at approximately 30-34°C were pipetted over the surface of the inoculated HIA plates. Once the overlay media solidified, a 100 µl aliquot of a saline suspension containing approximately 10^6 CFU of wild type or the catalase deficient mutant was spread onto the overlay. After incubation at 37°C in 5% carbon dioxide for

20-24 hours, the overlays were examined for zones of growth inhibition above the lactobacillus growth. The diameters of both the zone of gonococcal growth inhibition and the lactobacillus growth were measured, and a ratio of the two diameters was calculated to allow for standardization of the results despite differing sizes of lactobacillus growth. In some assays, bovine catalase (Worthington Biochemicals; Lakewood, NJ) was incorporated in the overlay media at varying concentrations (0 to 1,000 units/ml.

III. *In vitro* Growth Characteristics of Relevant *Lactobacillus spp.* Sets of 25 test tubes containing 3 ml L-MRS broth were inoculated with *L. crispatus, L. jensenii*, or *L. murinus* to establish an initial OD_{600} of 0.05 (time zero), and incubated at 37°C in 5% carbon dioxide under identical conditions. At selected time intervals between 0 and 76 hours, a tube was removed from the incubator and analyzed for OD_{600}, pH, lactate concentration, and the number of CFU/ml.

IV. Hydrogen Peroxide Production. The ability of lactobacilli to produce hydrogen peroxide was assessed using the qualitative agar assay described by McGroarty et al (1992). Briefly, lactobacilli were streaked onto L-MRS agar containing 0.25 mg/ml tetramethylbenzidine (Sigma Diagnostics; St. Louis, MO) and 0.01 mg/ml horseradish peroxidase (Sigma Diagnostics; St. Louis, MO). For best results, media were prepared three days prior to analysis. The plates were then incubated in an anaerobic culture jar (Key Scientific Products; Round Rock, TX) at 37°C for 48 hours. Following incubation, the plates were exposed to ambient air, which initiated the horseradish peroxidase to oxidize the tetramethylbenzidine if hydrogen peroxide was present. The presence of a

blue pigment in the colonies was interpreted as being positive for hydrogen peroxide production. An example is shown as Figure 4.

V. Molecular Identification of *Lactobacillus spp.* The polymerase chain reaction (PCR) assay described by Song et al (2000) was utilized to confirm the identity of lactobacilli isolated from mice. The following DNA primers corresponding to distinct 16S-23S rRNA intergenic spacer regions for *L. crispatus* and *L. jensenii* were used. The primers for *L. crispatus* were Lcri-3 (5'-AGGATATGGAGAGCAGGAAT-3') and Lcri-2 (5'-CAACTATCTCTTACACTGCC-3'). Primers for *L. jensenii* were 23-10C (5'-CCTTTCCCTCACGGTACGT-3') and Ljen-3 (5'-AAGAAGGCACTGAGTACGGA-3'). Two representative colonies from each culture to be tested were lifted from the agar surface and suspended in 50 µl of Tris-HCl-EDTA-saline solution. The suspension was incubated at 95°C for 10 minutes, and then centrifuged for 3 minutes at 12,000 rpm. The supernatant was used as the source of the DNA template. Several formulations of the PCR-reaction mixture were tested to optimize the reaction. The optimal reaction consisted of 15.1 µl PCR-grade water, 1.0 µl of 0.15 U Taq DNA polymerase (Promega Corporation; Madison, WI), 3.0 µl of reaction buffer (Promega Corporation), 0.6 µl of a mixture containing 200 µM each of dATP, dCTP, dGTP, and dTTP (New England Biolabs, Inc.; Beverly, MA), 5 µl of 10-pmol primer mix containing each primer, and 3.0 µl of colony lysate supernatant. The PCR amplification was performed using a Perkin-Elmer DNA Model Cetus thermal cycler (Perkin-Elmer Corporation; Norway, CT). The thermal cycler program consisted of 35 cycles composed of a 20 second denaturation step (95°C) and a 2 minute annealing/extension step (55°C). A final 5 minute extension step at 74°C was incorporated at the end of the program. PCR products were separated on

0.8% agarose gels and stained with ethidium bromide. The resultant PCR products for *L. jensenii* and *L. crispatus* were 700 bp and 522 bp respectively. The specificity of the primers was confirmed by testing a collection of six lactobacillus strains in our laboratory (data not shown).

VI. Experimental Murine Genital Tract Infection. A modification of an experimental murine gonococcal genital tract infection model (*Jerse*, 1999) was utilized to test the ability of lactobacilli to protect against *N. gonorrhoeae* colonization. Pilot studies to first test the ability of *L. crispatus, L. jensenii, and L. murinus* to colonize the mice were performed as follows. Female BALB/c mice (17 to 22 g) were obtained from the National Cancer Institute (Bethesda, MD) and housed in filter top isolator cages containing autoclaved water, food, and litter. Following a two-week acclimation period to promote cessation of cycling, as explained by the Lee-Boot Effect (*Jacoby and Fox*, 1984), a cytological examination of stained vaginal smears was performed to determine the estrous cycle stage for each mouse (*Snell*, 1941). Only mice in the diestrus or anestrus stages were selected for further study. A 5 mg, 21-day controlled release estradiol pellet (Innovative Research of America; Sarasota, FL) was implanted into the mice intradermally. The enhanced growth of commensal flora, which occurs under the influence of estrogen, was controlled with streptomycin sulfate (0.1 mL of 24 mg/ml streptomycin sulfate given twice daily via intraperitoneal injection) and trimethoprim sulfate (0.04 g/100 ml of drinking water). Two days after implantation of the estradiol pellet, mice were colonized intravaginally with lactobacilli by pipetting 20 μL containing approximately 10^7 CFU of *L. crispatus, L. jensenii, or L. murinus* suspended in 28 mM 2[N-Morpholino]ethanesulfonic acid (MES) buffer (pH 6.7) (Sigma Diagnostics) directly

into the vaginal lumen. Mice in control groups received 20 µL MES buffer as a placebo. Immediately after intravaginal inoculation, mice are held inverted for 45 seconds to minimize loss of bacterial suspension from the vagina. Lactobacilli used to generate the inoculum suspension were obtained as follows. Lactobacilli were harvested from L-MRS agar after 22 hours of incubation using a sterile cotton swab, and inoculated into 20 ml of MRS broth, which was then incubated at 37°C in 5% carbon dioxide with a loose cap overnight. In order to obtain lactobacilli in mid-logarithmic phase growth, 1 ml of the overnight culture was inoculated into 10 ml of fresh L-MRS broth. After six hours of incubation (OD_{600} between 0.7 and 1.0), the bacteria were washed twice with sterile 28 mM MES, and then concentrated to achieve the highest concentration of lactobacilli possible (approximately 10^7 to 10^9 CFU/20 µl). The number of viable lactobacilli in the inoculum was determined by a standard serial dilution culture method.

For *in vivo* protection studies mice were treated with estradiol and antibiotics, and then inoculated with *L. crispatus* or buffer placebo as above. Two doses of gonococci were tested in separate experiments to determine if the concentration of gonococcal challenge affected pathogen recovery in the presence and absence of *L. crispatus*. For each experiment, the following four treatment groups were established, each consisting of eight mice, all of which were maintained under identical experimental conditions. The four treatment groups of mice used were: (1) mice colonized with *L. crispatus* and challenged with *N. gonorrhoeae* FA1090, (2) mice only challenged with *N. gonorrhoeae* FA1090, (3) mice colonized with *L. crispatus* and challenged with *N. gonorrhoeae* 24.2, and (4) mice only challenged with *N. gonorrhoeae* 24.2. Three hours after inoculation with lactobacilli or placebo, mice were challenged with 20 µl of wild-type (FA1090) or

catalase mutant (24.2) suspended in phosphate buffered saline containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS with $Ca^{2+}Mg^{2+}$). The gonococcal suspension was made by harvesting gonococci from GC agar (approximately 22 hours old) with a sterile cotton swab and suspending the bacteria in sterile PBS with $Ca^{2+}Mg^{2+}$. The suspension was filtered (1.2 μm pore size) (Gelman Sciences; Ann Arbor, MI) to remove gonococcal aggregates, and brought to an OD_{600} of 0.08, which was previously shown to correspond to a concentration of 5 x 10^7 CFU/ml. The number of viable gonococci in the inoculum was determined by a standard serial dilution culture method.

In the two mouse experiments in which the capacity of *L. crispatus* to inhibit *N. gonorrhoeae in vivo* was evaluated, a booster inoculum of lactobacilli was given to maintain the highest possible concentration of *L. crispatus* within the murine lower genital tract based on the observed decline in lactobacillus recovery from mice over time during pilot mouse studies. Booster inoculum was prepared in the same manner as described above for the initial inoculum. In the first experiment, a booster was given on day 5 (1.5 X 10^7 CFU/mouse) and day 10 (3.1 X 10^7 CFU/mouse). The mice in the second experiment received a booster only on day 5 (2.2 X 10^7 CFU/mouse).

Colonization by *N. gonorrhoeae* and/or *Lactobacillus spp.* was monitored by vaginal culture every even numbered day following inoculation through 14 days, after which the mice were sacrificed. For pilot vaginal pH studies, mice were cultured on odd numbered days following inoculation through day 13. Culturing consisted of gently inserting a sterile, saline moistened Dacron UltraMicroPur™ superfine swab (0.025 diameter) (Purfyber, Inc.; Munster, IN) into the vagina and slowly rotating it 180°. The swab was removed and immediately used to inoculate a small portion of the vaginal

mucus on HIA to detect the presence of facultatively anaerobic commensal organisms, and then smeared onto a sterile glass microscope slide. The remaining swab sample was suspended in 100 µL of PBS with $Ca^{2+}Mg^{2+}$. The swab suspension was then serially diluted 1:10 in GC broth containing 0.05% saponin to disrupt aggregated gonococci. For the lactobacillus pilot studies, a 25 µl aliquot of each dilution was cultured onto L-MRS agar. For the *in vivo* protection studies, 25 µl aliquots of the undiluted and diluted vaginal swab suspensions were cultured onto both L-MRS and supplemented GC agar containing vancomycin, colistin, nystatin, trimethoprim sulfate (VCNT supplement) (Becton Dickinson; Cockeysville, MD) and 100 µg/ml of streptomycin sulfate. The remaining swab suspension was frozen, and used for vaginal lactate determination (method described below).

VII. Measuring Lower Genital Tract Inflammation and Lactobacilli Adherence. Vaginal smears prepared in parallel with each culture were fixed and stained with a modified Wright-Giemsa stain (Heme-3) (Biochemical Sciences, Inc.; Swedesboro, NJ). The stained smears were viewed by light microscopy to determine the cellular composition of the superficial layers of the vaginal mucosa. The percent of neutrophils (PMN), squamous epithelial cells, and nucleated epithelial cells was determined by classifying 100 consecutive vaginal cells. Inflammation was defined as two consecutive time-points in which the percentage of PMNs was greater than 25%, based on previous experiments in which fewer than 25% PMNs was the baseline level in uninfected, placebo control mice, and therefore, considered non-inflammatory (*Jerse,* 1999). In pilot studies, the adherence of lactobacilli to vaginal epithelial cells was also evaluated. The average number of lactobacilli per epithelial cell was determined by viewing 100

epithelial cells. The degree of adherence by different lactobacilli strains was compared, and used as a criterion in the selection of the best probiotic candidate.

VIII. Determination of Vaginal pH. Vaginal mucosal pH was measured using a 16-gauge MI-414-E (2 cm length) combination pH microelectrode (Microeletrode, Inc; Bedford, NH). Following calibration, the pH probe was cleansed with a sterile 70% isopropyl alcohol pad and rinsed in sterile normal saline before insertion into the vagina of each mouse. Care was taken to minimize disruption of the mucosa during insertion. Readings were measured in duplicate for each mouse 1-hour prior to bacterial culture.

IX. Determination of Lactate Levels. Due to the production of lactate by *Lactobacillus spp,* and its role in acidification, the presence of lactate was measured during *in vitro* and *in vivo* growth of lactobacilli. For the *in vitro* studies, the concentration of lactate in spent culture media was determined using a quantitative enzymatic procedure (Sigma Diagnostics procedure 826-UV; St. Louis, MO). The specimens were deproteinized by adding 0.2 ml of specimen to 0.4 ml of 10% (volume/volume) tricholoroacetic acid (Sigma Diagnostics) as per manufacturer's instruction. The mixture was vortexed for 30 seconds, and then placed at 2°-8°C for 10 minutes. The precipitated protein is removed from solution by centrifuging the specimen at 12,000 rpm for 10 minutes. Once the specimen was deproteinated, 0.1 ml was added to a cuvette containing the reaction reagents and mixed gently by inversion. The cuvettes were incubated for 15 minutes at 37°C. Absorbance was read at 340 nm, and lactate concentration was calculated from a standard curve. A standard curve and controls were used for each lactate determination. Lactate analysis was also performed as described above on pooled vaginal swab suspensions from mice in the same experimental test group due to the sample

requirement of 0.2 ml/test. Although the use of pooled samples was not ideal, this allowed us to determine if lactate was being produced *in vivo*.

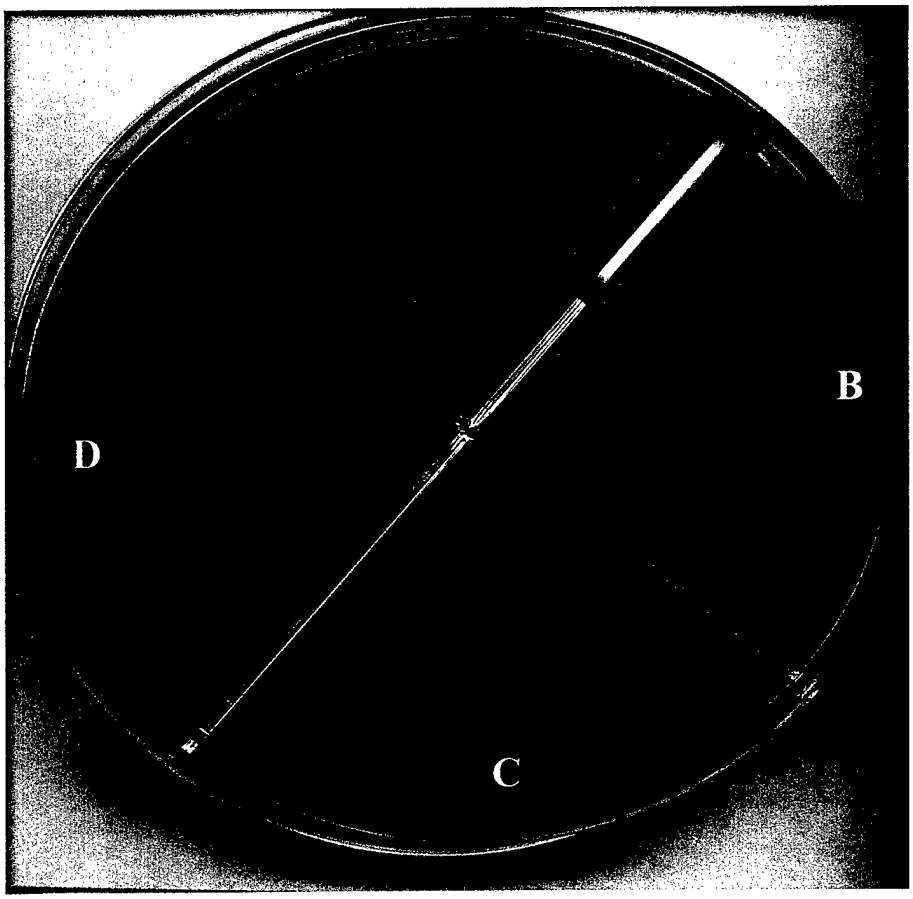
X. Statistical Analysis. The statistical software SPSS 11.0™ (SPSS Sciences; Chicago, IL) was utilized to analyze the data as needed. Specifically, the student paired T-test was used to assess the significance of duration of infection and a repeated ANOVA was used to compare the number of gonococci recovered from *in vivo* experimentation treatment groups. *P* values of <0.05 were considered significant.

RESULTS

I. Gel Overlay Inhibition Studies. A gel overlay inhibition assay was utilized to compare the capacity of *L. crispatus*, *L. jensenii*, and *L. murinus* to inhibit *N. gonorrhoeae* and to identify the responsible inhibitory factor(s). As already discussed in Materials and Methods, *L. crispatus* and *L. jensenii* both have the ability to produce hydrogen peroxide, while *L. murinus* does not (Figure 4).

a. ***In vitro inhibition of N. gonorrhoeae FA1090 and catalase-deficient mutant 24.2 by human vaginal Lactobacillus strains.*** Both *L. crispatus* (ATCC 33197) and *L. jensenii* (ATCC 25258) inhibited *N. gonorrhoeae* strain FA1090 at neutral (7.2) and acidic pH (5.8). The amount of inhibition was quantified as described in Materials and Methods and is shown in Figure 5. The degree of inhibition conferred by *L. crispatus* was greater than that by *L. jensenii* at both neutral and acidic conditions based on the larger zone of inhibition above the spot inoculum of *L. crispatus*. An additional notable observation was that the inhibition of gonococci by *L. crispatus* was enhanced under acidic conditions. Conversely, the gonococcal inhibition by *L. jensenii* was lessened when the media was acidified, and the zone of inhibition produced *by L. jensenii* at acidic pH was smaller in diameter than that of the lactobacillus spot inoculation (ratio of approximately 0.84). *L. murinus*, a murine lactobacillus isolate (strain LB10A) did not inhibit *N. gonorrhoeae* under any conditions tested (data not shown). The increased inhibition by lactobacilli at acidic pH may be due to the fact that acidity stabilizes hydrogen peroxide, and thus, increases the inhibition (*Fontaine and Taylor-Robinson*, 1990). An alternative explanation is that lactobacilli may produce more hydrogen peroxide at acidic conditions.

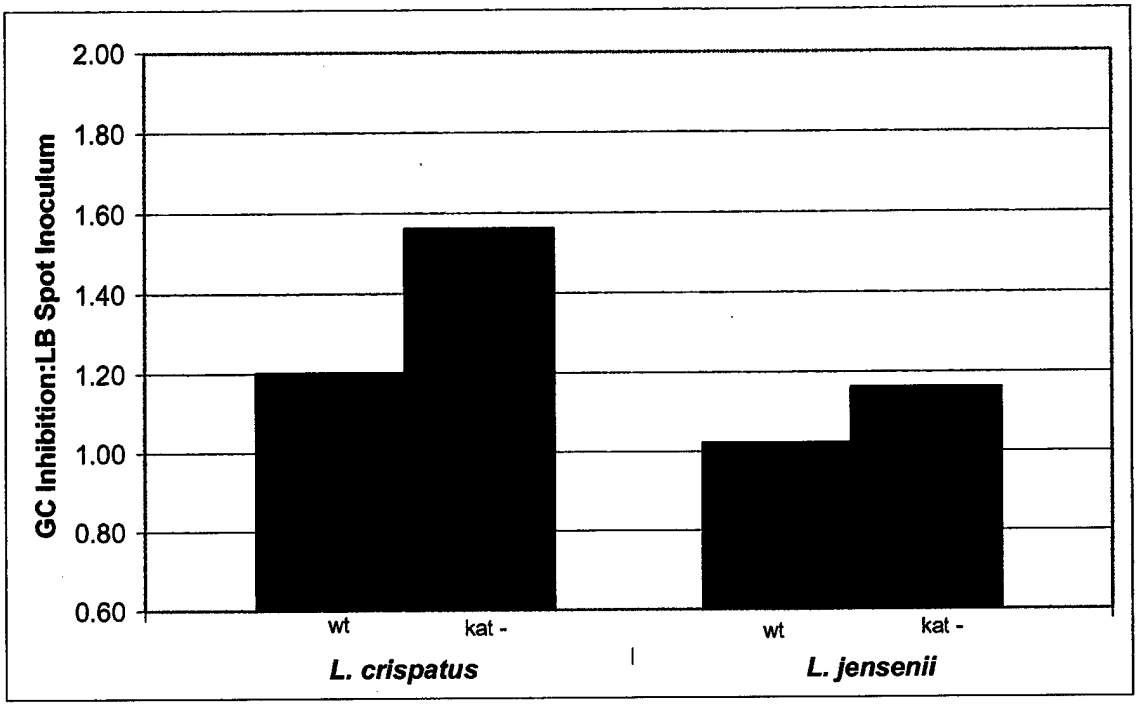
Figure 4. Detection of hydrogen peroxide production by *L. crispatus* and *L. jensenii* using the agar assay described by McGroarty et al (1992). A, no bacteria; B, *L. murinus*; C, *L. crispatus;* and D, *L. jensenii.*



The inhibition of the catalase-deficient mutant 24.2 by *L. crispatus* and *L. jensenii* was reproducibly increased as compared to wild-type gonococci (Figure 6). As illustrated in Figure 6B and 6D, inhibition of strain 24.2 by lactobacilli was observed directly above the undiluted (standard) and 10^{-1} dilutions of *L. crispatus* and *L. jensenii*. In contrast, inhibition of the wild-type strain only occurred with the undiluted (standard) lactobacillus inocula (Figures 6A and 6C).

Figure 5. Histogram representation of the inhibition of wild-type (wt) and catalase-deficient mutant (*kat-*) *N. gonorrhoeae* by *L. crispatus* and *L. jensenii* at pH 7.2 (A) and pH 5.8 (B). Overlays were performed in triplicate, and the results represent the average inhibitions. The experiment was performed twice, and the results were similar.

A.



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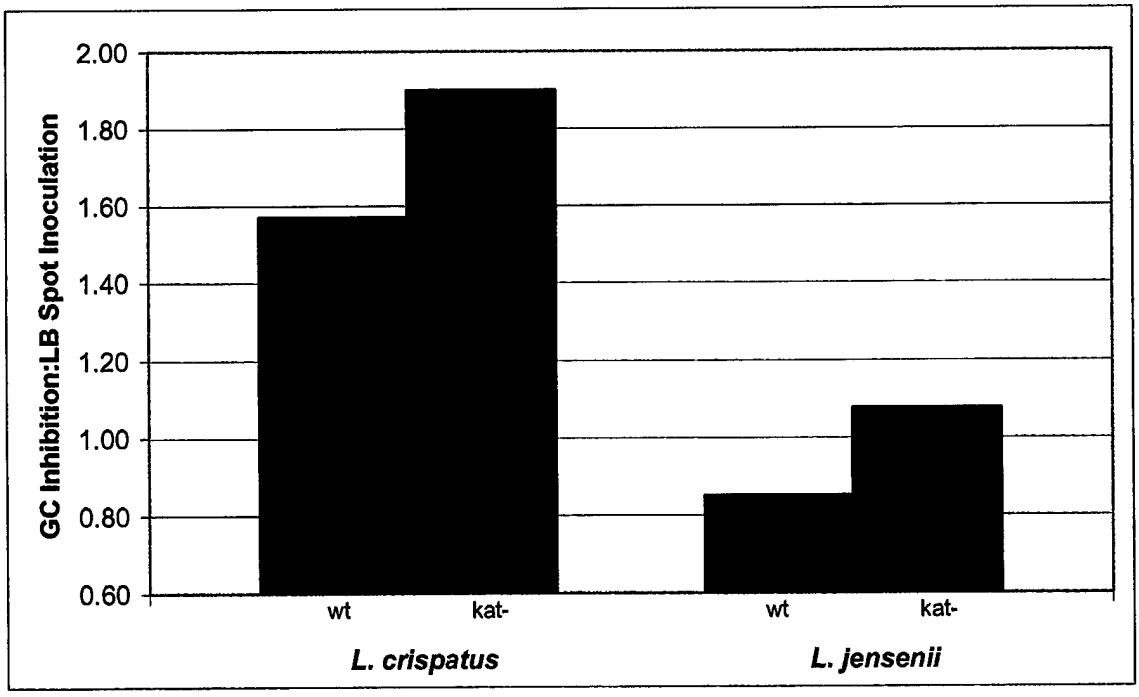
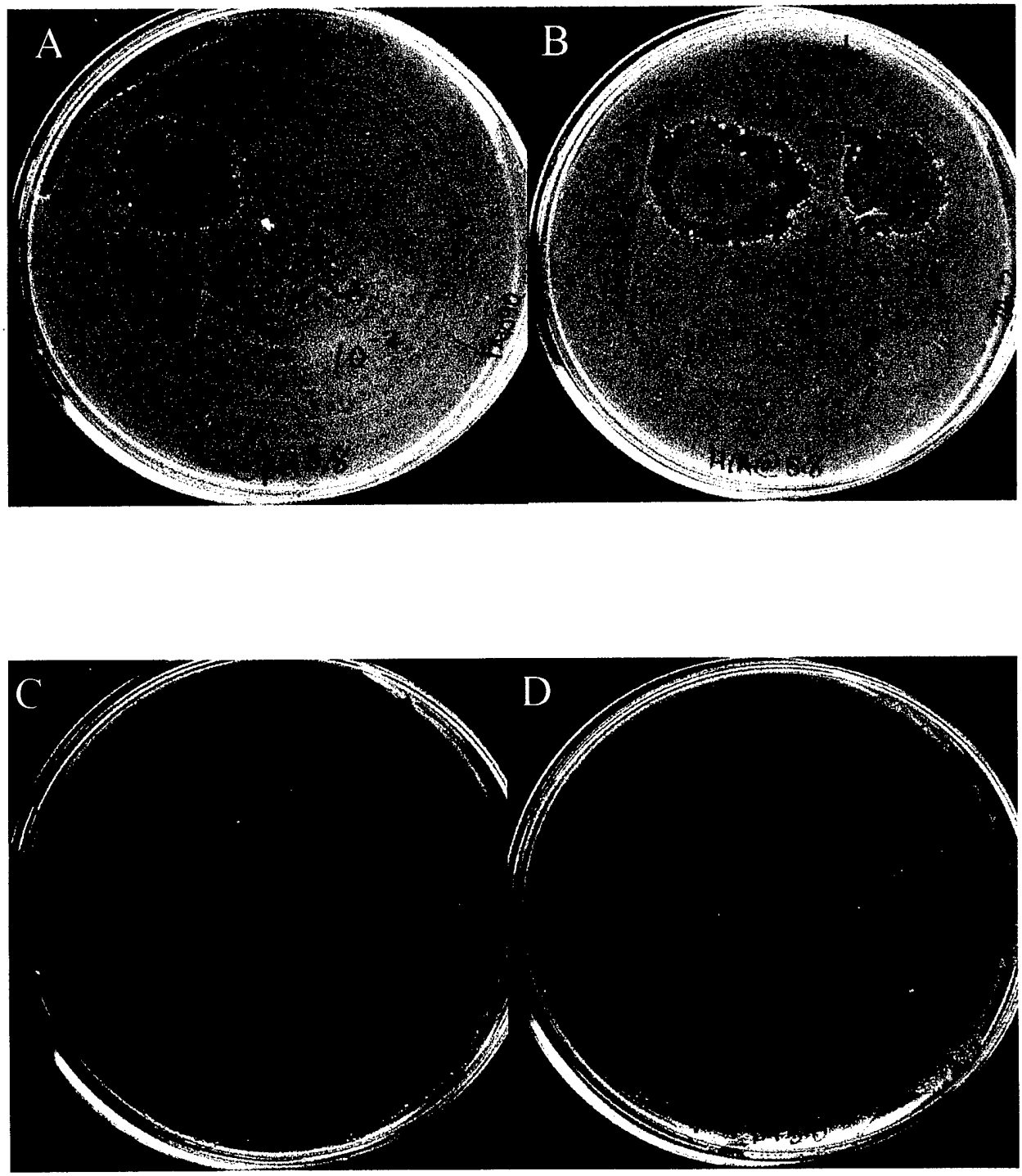


Figure 6. Inhibition of wild-type (FA1090) (A,C) and catalase-deficient *N. gonorrhoeae* (24.2) (B,D) by *L. crispatus* (A,B) and *L. jensenii* (C,D) cultured at acidic pH. Zones of inhibition appear as clearings above the lactobacilli spot inoculations.



b. ***Neutralization of Lactobacillus Inhibition with Exogenous Catalase.*** To investigate the mechanism by which *L. crispatus* and *L. jensenii* inhibit *N. gonorrhoeae in vitro*, bovine catalase was incorporated in the overlay media. Gonococcal inhibition by *L. crispatus* and *L. jensenii* at neutral pH was neutralized by the presence of 5 units/mL of exogenous catalase (Table 2). However, a ten-fold increase in exogenous catalase was required (50 units/mL) to neutralize the inhibition of gonococci by *L. crispatus* at acidic pH (Figure 7 and Table 2). The amount of catalase required to neutralize gonococcal inhibition by *L. jensenii* at acidic conditions was unchanged (5 units/mL). These results are consistent with the higher level of inhibition of *N. gonorrhoeae* by *L. crispatus* at acidic pH (Figure 7B and 7C). *L. murinus*, which was utilized as a negative control, showed no inhibition of *N. gonorrhoeae* FA1090 in the presence and absence of bovine catalase. Ten-fold more catalase compared to the amount needed to neutralize the inhibition of wild-type *N. gonorrhoeae* was required to neutralize the inhibition of the catalase mutant 24.2 by the respective lactobacilli (data not shown). These results suggest gonococcal inhibition by lactobacillus is mediated primarily by hydrogen peroxide. Furthermore, the fact that neutralization by bovine catalase was dose-dependent suggests that *L. crispatus* produces a higher amount of hydrogen peroxide than *L. jensenii*.

Figure 7. Gel overlays containing exogenous bovine catalase in the overlay agar. The capacity of three strains of lactobacilli (LJ, *L. jensenii*; LC, *L. crispatus*, LM, *L, murinus*) to inhibit *N. gonorrhoeae* FA1090 at pH 5.8 in the presence of bovine catalase at the following concentrations was assessed. A, 50 catalase units/mL; B, 5 catalase units/mL; C, no catalase.

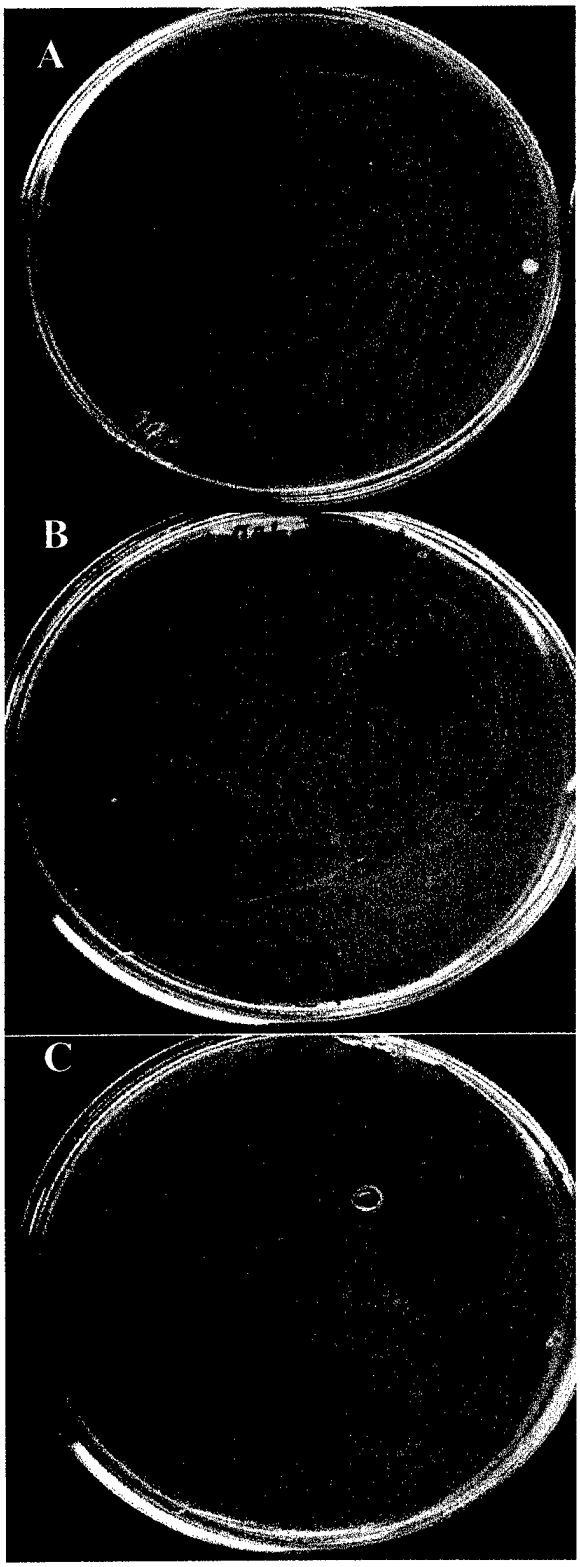


Table 2. Neutralization of lactobacillus inhibition of *N. gonorrhoeae* FA1090 by the addition of exogenous catalase under neutral and acidic culture conditions.

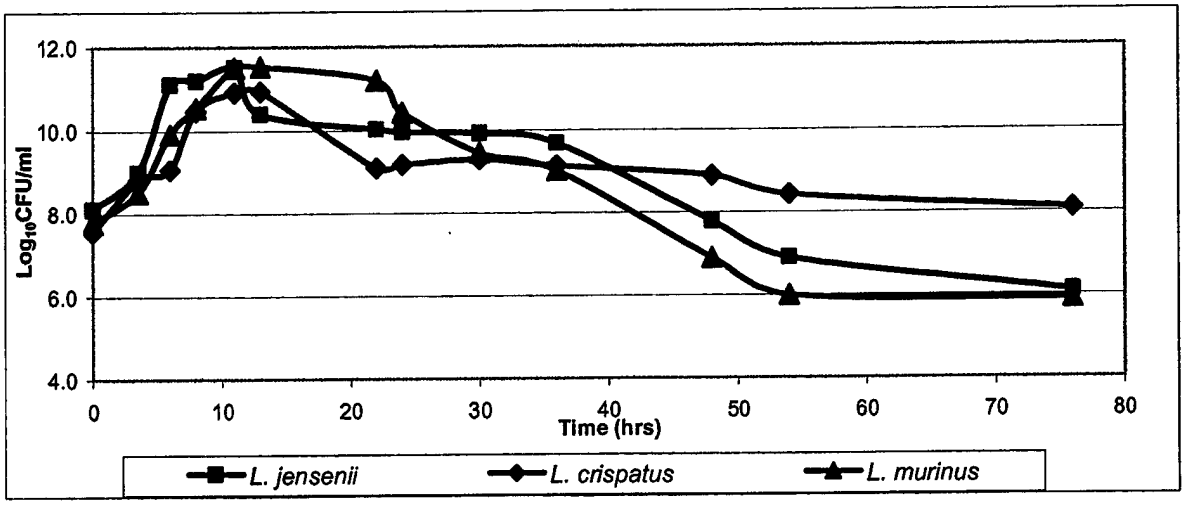
	[Catalase] in Overlay Media (U/mL)				
Lactobacillus spp.	**1000**	**500**	**50**	**5**	**0**
L. jensenii (pH 7.2)	**No Inhibition**	**No Inhibition**	**No Inhibition**	**No Inhibition**	
L. crispatus (pH 7.2)	**No Inhibition**	**No Inhibition**	**No Inhibition**	**No Inhibition**	
L. jensenii (pH 5.8)	**No Inhibition**	**No Inhibition**	**No Inhibition**	**No Inhibition**	
L. crispatus (pH 5.8)	**No Inhibition**	**No Inhibition**	**No Inhibition**		

II. *In vitro growth characteristics of Lactobacillus spp.* To generate more information on the potential of *L. crispatus* and *L. jensenii* to protect against *N. gonorrhoeae in vivo*, we measured the acidification of L-MRS broth cultures by *L. crispatus*, *L. jensenii*, and *L. murinus* as well as lactate production and bacterial viability over time as described in Materials and Methods. *L. jensenii* and *L. murinus* LB10A proliferated at similar rates, while *L. crispatus* demonstrated a slower growth rate (Figure 8A). The growth phases of each lactobacillus strain as defined by CFU recovered over time are presented. The logarithmic growth phase of the lactobacilli occurred between 3.5 hours and 11 hours. Stationary phase began at approximately 11 hours and by 22 hours death phase was detected.

The lactate concentration and the pH of the growth media were also plotted as a function of time (Figure 8B). In all cases, as the lactobacillus load increased, the pH of the media became more acidic with the initial pH of 5.7 decreasing to 3.5 during the course of a 76-hour experiment. All three strains acidified the broth at relatively similar rates. The concentration of lactate increased with time, and correlated with the decrease in pH (Figure 8C). The total concentration of lactate per viable bacterium was greatest for *L. crispatus*. During the logarithmic phase, *L. crispatus* produced lactate at a rate of 2.1 mmol/L/hour. Appreciable concentrations of *L. crispatus*-derived lactate were detected at 6 hours, consistent with mid-logarithmic phase growth. Lactate was detected in broth cultures of *L. crispatus* and *L. murinus* LB10A as early as 3.5 hours. Surprisingly, lactate produced by *L. jensenii* was not detected until 11 hours (stationary growth phase). A possible explanation of this result may be that *L. jensenii* is heterofermentative, and thus, produces acidic by-products in addition to lactate (i.e., formate, succinate).

Figure 8. *In vitro* growth rates (A), growth media acidification (B), and lactate production (C) of *L. jensenii, L. crispatus, and L. murinus* in L-MRS broth. The experiment was performed twice and the results were similar.

A.



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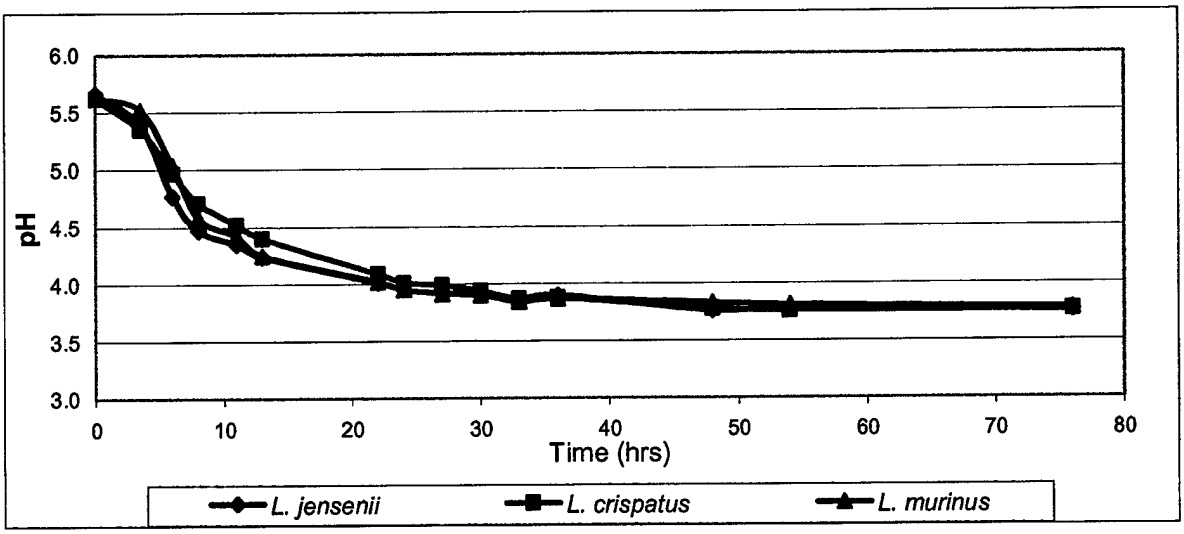
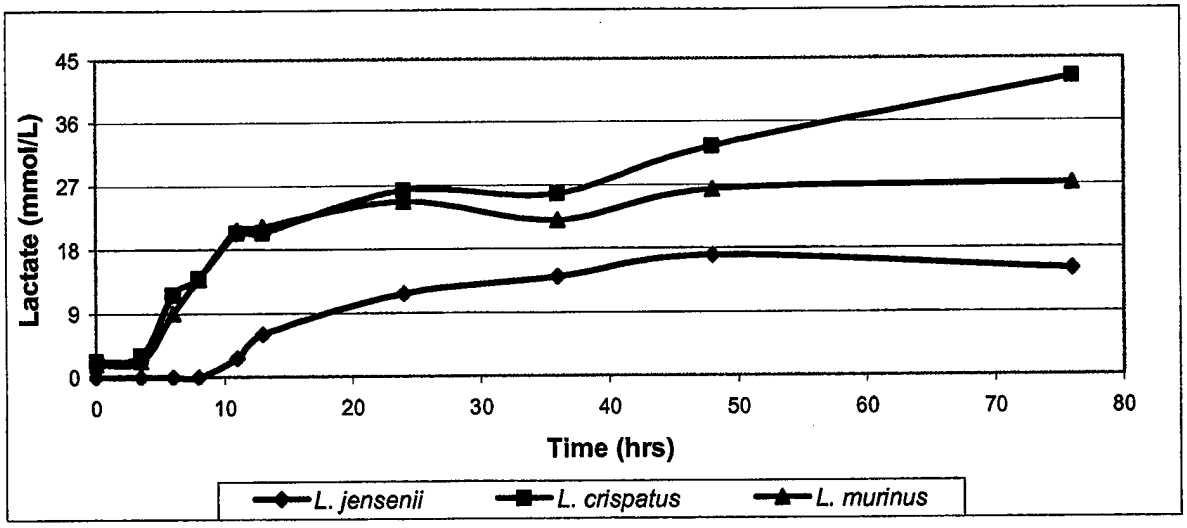


C.



III. *Pilot mouse lactobacillus colonization studies.* *In vitro* studies revealed *L. crispatus* to be the best candidate to inhibit *N. gonorrhoeae in vivo* based on its ability to produce the highest concentrations of lactate when cultured in L-MRS broth and hydrogen peroxide as observed indirectly by gel overlay assays. Because colonization is crucial to the success of *in vivo* studies, pilot studies were designed to compare the ability of *L. jensenii* and *L. crispatus* to colonize the lower genital tract of estradiol-treated mice. *L. murinus* strain LB10A was utilized as control.

a. ***Colonization***. Previous research conducted in our laboratory showed long-term colonization of mice with human lactobacillus isolates was maximized by treating BALB/c mice with estradiol 48 hours prior to inoculation with lactobacilli (*St. Amant and Jerse*, personal communication). Therefore, estradiol-treated BALB/c mice were used in this study. Mice were inoculated with *L. crispatus*, *L. jensenii*, or *L. murinus* as described in Materials and Methods, and the lower genital tract was cultured for 14 days. The duration of colonization by each lactobacillus strain tested is shown in Table 3. Surprisingly, there was no statistical difference in the duration of recovery from mice colonized with any of the three strains. The mean duration of colonization by *L. jensenii* and *L. crispatus* from the genital tract of BALB/c mice was greater than 1 week in each case. There was also no statistical difference in the number of lactobacilli recovered over time.

The adherence of the three lactobacilli strains to vaginal epithelial cells was also assessed to better understand if colonization was a function of adherence, and/or biofilm formation. The average number of lactobacilli adhering to epithelial cells from mice in each group on days 1 through 5 after inoculation was estimated (Table 4). These time points were chosen because the lactobacillus load was most stable early in infection.

There was no significant statistical difference between the degrees of epithelial adherence of the three lactobacillus strains to mouse epithelial cells. Interestingly, many notable "bacterial sheets/films" were observed, suggestive of coaggregation (biofilm) between bacteria.

Table 3. Recovery of lactobacilli isolates from estradiol-treated mice inoculated intravaginally with *L. crispatus*, *L. jensenii*, or *L. murinus*.

Lactobacilli	Mean Duration of Recovery[†] (Range)
L. crispatus	8.2 days (1 - 11)
L. jensenii	9.8 days (1 - 13)
L. murinus	12.2 days (1- 13)

[†] Limit of detection: 4 CFU/100 µl vaginal swab suspension.

Table 4. Adherence of lactobacilli to murine lower genital tract epithelial cells.

Lactobacilli	Average Number of Lactobacilli/Cell (range)
L. crispatus	1.49 (0.91 - 1.98)
L. jensenii	1.52 (0.80 - 2.08)
L. murinus	1.93 (1.15 - 2.36)

b. ***Vaginal pH.*** A second pilot study was conducted to determine the effect of lactobacillus colonization on the physiology of the murine lower genital tract. This study included five treatment groups consisting of 3 to 5 mice per group. Four of the treatment groups included mice treated with estradiol followed by intravaginal inoculation with approximately 10^9 CFU of *L. crispatus*, *L. jensenii*, *L. murinus,* or MES-placebo control. A fifth group consisted of mice not treated with estradiol or given bacteria. Bacterial load, vaginal pH, lactate concentrations, and inflammation were monitored as described in Materials and Methods.

Mice that did not receive estradiol or lactobacillus had an average vaginal pH of 6.77 (range of 6.38 to 7.01). Interestingly, mice that received estradiol and no lactobacilli had an average vaginal pH of 6.34 (range of 5.72 to 7.25). Therefore, from the data obtained from mice that were not experimentally inoculated with lactobacilli, it appears that vaginal pH was dependent on estradiol. Exclusion criteria were utilized to better analyze the data. Three mice that did not receive estradiol and from which no naturally occurring *L. murinus* was recovered had a vaginal pH of 6.79 (range of 6.38 to 7.01), a result consistent with Braude et al (1978). The vagina of one mouse not receiving exogenous estradiol or lactobacilli became naturally colonized with the murine strain of lactobacilli on day 7 of the experiment (Figure 9). The average vaginal pH of this mouse was 6.66 (6.38 to 6.93). Four mice that received estradiol and were not naturally colonized with *L. murinus* had an average pH of 6.32 (range of 5.72 to 7.25). The remaining mouse in this group had an average vaginal pH of 6.45 (6.12 to 6.94).

Analysis of data from mice that were experimentally challenged with the 3 lactobacillus strains revealed no significant difference between lactobacillus strains and vaginal pH. The average vaginal pH of mice possessing *L. crispatus*, *L. jensenii*, and *L.*

murinus over the 13 day period was 6.31, 6.43, and 6.51 respectfully. To control for differences in colonization load and to determine if vaginal pH was dependent on the number of lactobacilli, exclusion criteria based on the number of lactobacilli recovered at each time point were implemented (Table 5). No clear or dramatic decrease in pH was associated with high lactobacillus loads for mice inoculated with *L. crispatus* and *L. murinus*. Mice inoculated with *L. jensenii* at levels greater than 1 x 10^6 CFU/100 µl had the lowest murine vaginal pH at these time points (Table 5). Recovery of *L. crispatus* and *L. jensenii* at a concentration greater than 5 x 10^5 lactobacillus CFU/100 µl vaginal swab suspension was associated with the lowest murine vaginal pH (Table 5). At no time point was the average vaginal pH < 5.68 for any mouse in the study. An analysis of individual mice inoculated with *L. crispatus* or *L. jensenii*, which plots vaginal pH versus number of lactobacilli recovered over time, is depicted in Figures 10 and 11. Overall, no clear association between vaginal pH and number of lactobacilli was detected. A potential association between vaginal pH and lactobacillus load was detected early in infection for 3 of 5 mice inoculated with *L. crispatus* (Figure 10, mice 9, 11, and 12) and 2 of 5 mice inoculated with *L. jensenii* (Figure 11, mice 16 and 17). In these mice, 1 x 10^5 CFU of the relevant lactobacillus were isolated on days 1 and 3, and the average vaginal pH was 5.95. However, this association was not usually found at later time points.

Overall, high numbers of *L. crispatus* or *L. jensenii* were recovered from the majority of mice over the first 5 days of the experiment. The range of lactobacilli recovery was approximately 10^4 to 10^6 CFU per 100 µl vaginal swab suspension. After 5 days, 70% of the mice had a major decrease in the number of vaginal lactobacilli. This observation prompted the need to provide a lactobacillus "booster" at day 5 during *in vivo*

protection studies to maintain the highest possible concentration of lactobacilli. In occasional mice (e.g., mice 13 and 14 shown in Figures 10 and 11, respectively), a transient decrease in recovery of *L. crispatus* and *L. jensenii* occurred on day 7. This observation may be explained by sampling error.

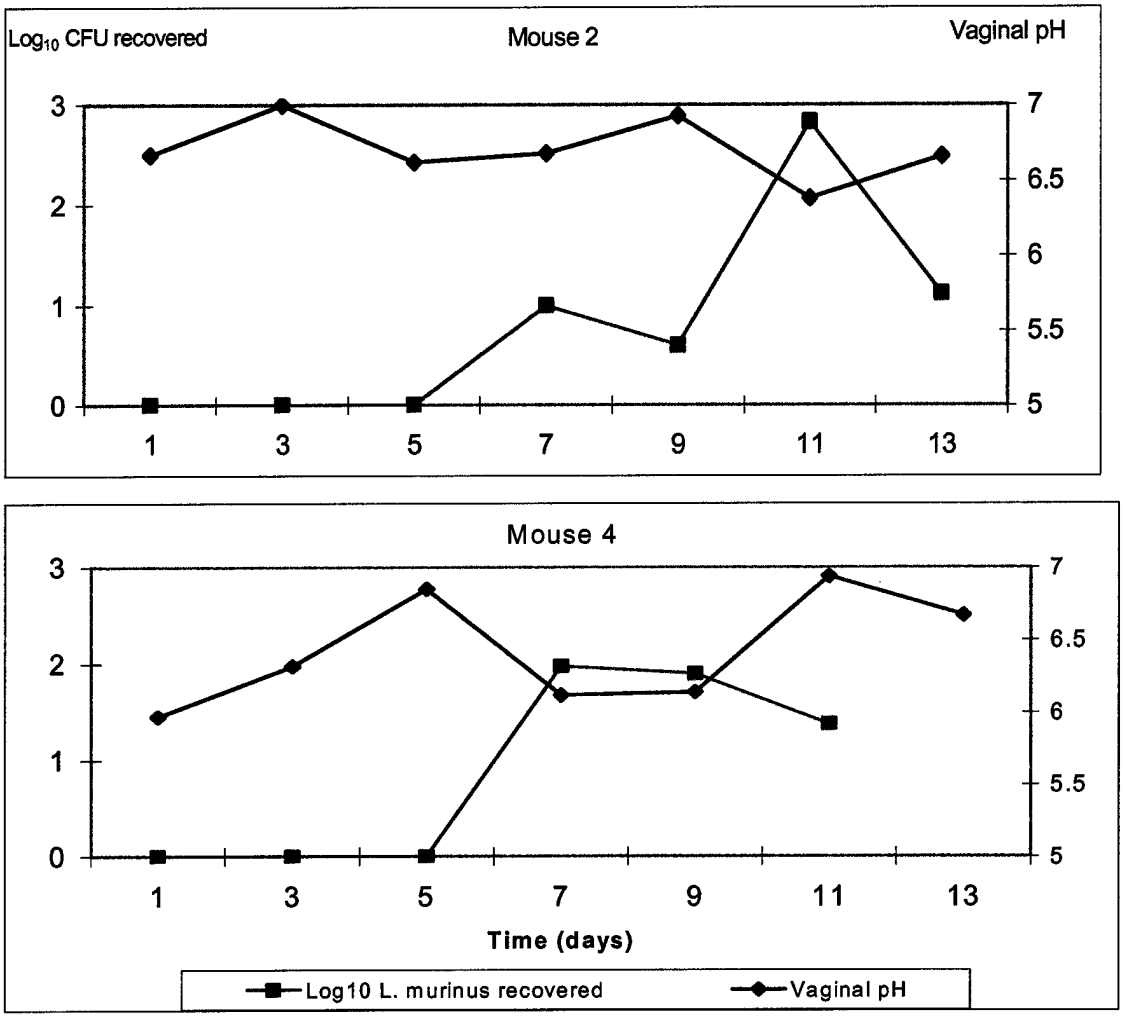
Our results are not consistent with data from *in vitro* and human studies in which vaginal acidity correlates with the concentration of vaginal lactobacilli (*Boskey et al*, 1999; *Sautter and Brown*, 1980). The vaginal pH of mice has been reported to be much less acidic, and to be slightly dependent on estrous stage (*Braude et al*, 1978). This study, however, did not account for normal flora load or type. Although the number of mice in the pilot experiment was small, even with deliberate vaginal inoculation with lactobacillus, we did not observe acidification. The absence of glucose and/or the presence of a unique murine buffering system in the vagina could account for the absence of acidity. It is also possible that higher levels of lactobacilli ($> 5 \times 10^6$ CFU/100 µl vaginal swab suspension) are needed to achieve greater acidification, and therefore, we could not duplicate the human vaginal pH in our mouse model. Our hope is that future studies will elucidate this mystery.

Table 5. Vaginal pH of mice inoculated with *L. crispatus, L. jensenii, and L. murinus* with respect to colonization levels.

	L. crispatus		*L. jensenii*		*L. murinus*	
Number of Lactobacilli Recovered	**Ave. pH (range)**	**n***	**Ave. pH (range)**	**n***	**Ave. pH (range)**	**n***
$\geq 1 \times 10^5$ CFU/100µl	6.31 (5.86-7.02)	27	6.43 (5.74-7.09)	31	6.51 (5.68-7.03)	31
$\geq 5 \times 10^5$ CFU /100µl	6.18 (5.86-6.72)	7	5.98 (5.74-6.17)	4	6.53 (5.97-6.97)	8
$\geq 1 \times 10^6$ CFU /100µl	6.31 (5.87-6.72)	4	5.98 (5.74-6.17)	4	6.77 (6.65-6.97)	5

* n denotes the total number of pH readings from which the stated amount of lactobacilli were recovered. Vaginal pH was measured 1 hour prior to vaginal culture.

Figure 9. Recovery of *L. murinus* and vaginal pH in an untreated mouse (Mouse 2) and an estradiol-treated mouse (Mouse 4) that were not challenged with lactobacillus over time. Both mouse 2 and 4 were naturally colonized by *L. murinus,* which was first detected on day 7. Yellow background designates mouse data that support the relationship between number of lactobacilli and vaginal pH.



† Limit of detection: 4 CFU/100 µl vaginal swab suspension.

Figure 10. Recovery of *L. crispatus* and vaginal pH over time. Yellow background designates mouse data supporting a relationship between number of *L. crispatus* and vaginal pH at early time points as described in the text. Limit of detection: 4 CFU/100 µl vaginal swab suspension.

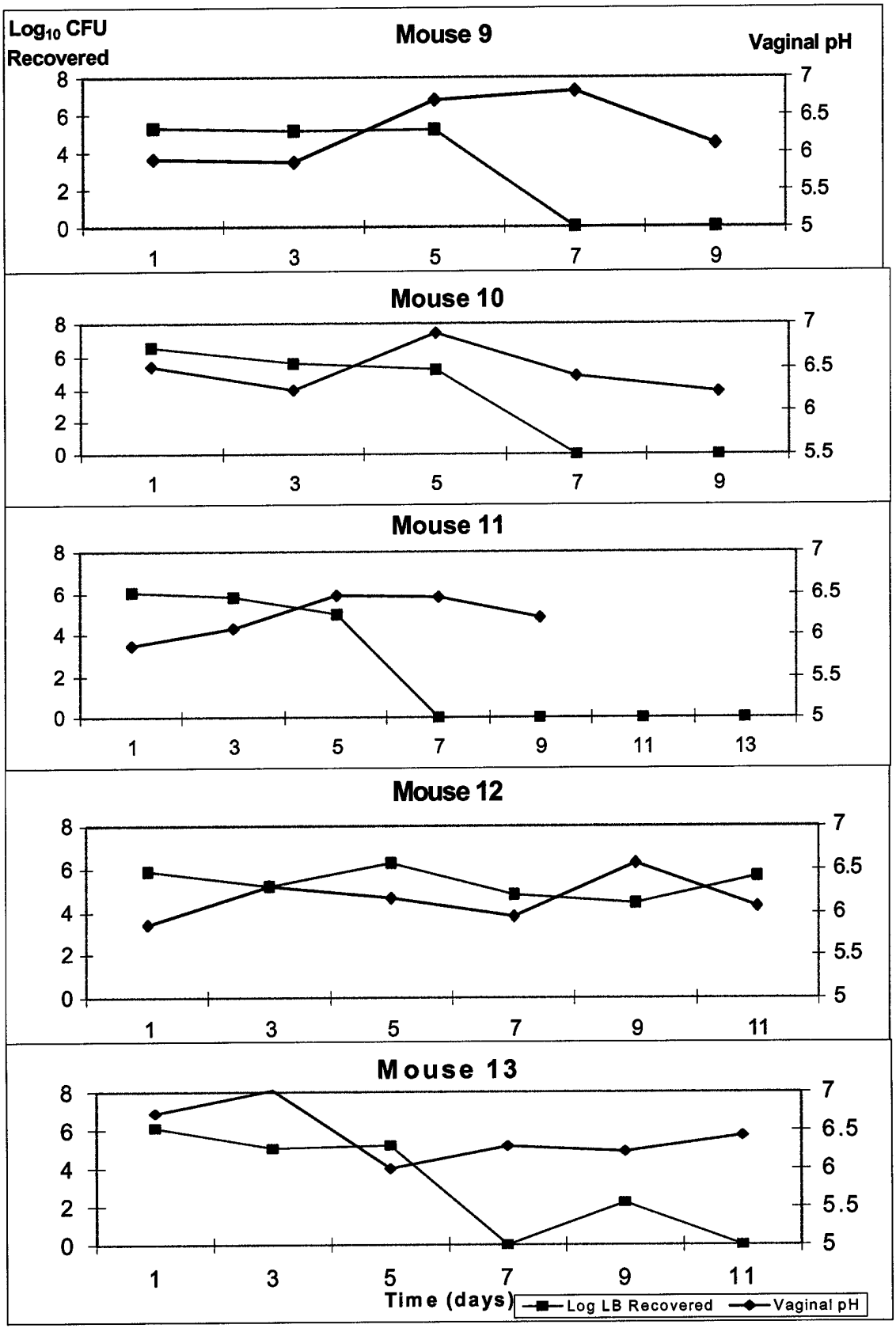
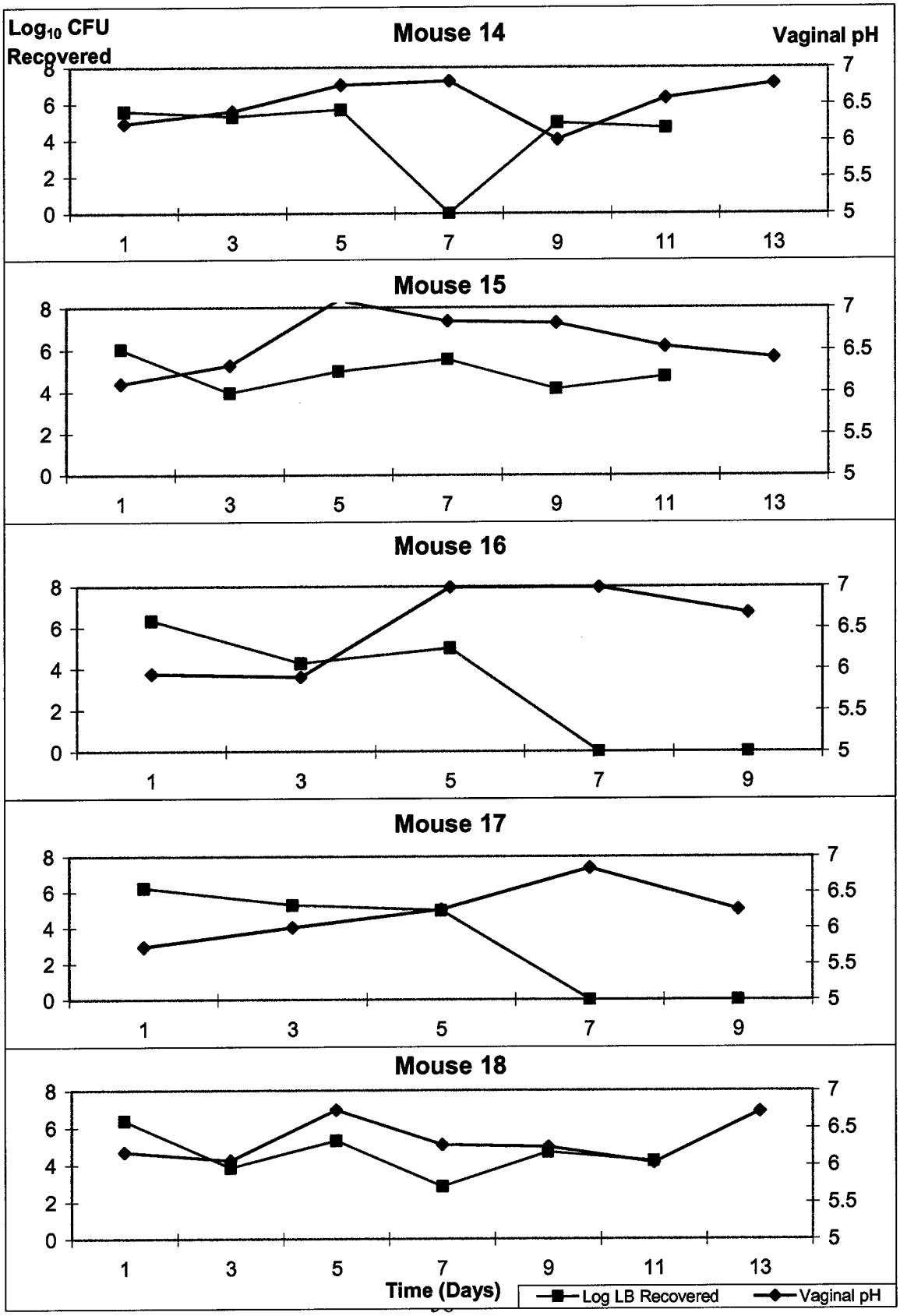


Figure 11. Recovery of *L. jensenii* and vaginal pH over time. Yellow background designates mouse data supporting a relationship between number of *L. jensenii* CFU recovered and vaginal pH at early time points as described in the text. Limit of detection: 4 CFU/100 µl vaginal swab suspension.



IV. ***In vivo Protection Studies using L. crispatus.*** *In vitro* and *in vivo* studies designed to assess the growth and inhibitory characteristics of *L. crispatus* and *L. jensenii* revealed no major differences in colonization and vaginal acidification. However, *in vitro*, *L. crispatus* inhibited gonococci more than *L. jensenii*. Consequently, we assessed the ability of *L. crispatus* to protect against experimental gonococcal infection of mice as described in Materials and Methods. Two gonococcal challenge doses were tested (10^6 and 5×10^5 CFU/mouse).

a. ***Experiment 1.*** Previous dose response experiments by Jerse et al (1999) showed an initial inoculum of 1×10^6 resulted in long-term recovery (> 5 days) of gonococci from 80% of estradiol-treated mice (ID_{80}); a dose of 1×10^5 caused long-term infection in only 50% of the mice. We therefore chose a challenge dose of 10^6 to test the protective potential of *L. crispatus*. Mice were inoculated with approximately 5×10^6 CFU *L. crispatus* and challenged with approximately 1×10^6 CFU of wild-type gonococci or 4×10^5 CFU of catalase-deficient gonococci. The lower dose of the catalase deficient strain was inadvertent, as a dose of 1×10^6 CFU was planned. Control groups were challenged with wild-type or catalase mutant *N. gonorrhoeae* alone. No significant difference in the duration of recovery of *N. gonorrhoeae* was detected between the four test groups (Table 6). The average number of wild-type or catalase-deficient *N. gonorrhoeae* over time in mice pre-colonized with *L. crispatus* versus the control group was also evaluated. As shown in Figure 12, there was no statistically significant difference in the number of *N. gonorrhoeae* FA1090 or 24.2 gonococci recovered from mice with *L. crispatus* and without *L. crispatus* as evaluated by repeated ANOVA.

L. crispatus was detected in all mice that were inoculated with *L. crispatus* through the course of the experiment. The identity of the lactobacillus isolates was presumptively made by colony morphology using a 10X stereomicroscope and hydrogen peroxide-production. To confirm that the lactobacilli isolated in vaginal cultures was *L. crispatus* and not *L. murinus*, PCR-analysis was used as described in Materials and Methods. All colonies tested gave the 522-bp PCR product, consistent with the identity of *L. crispatus* (Figure 13). The average recovery of *L. crispatus* is also shown in Figures 19A and 19B (dotted line).

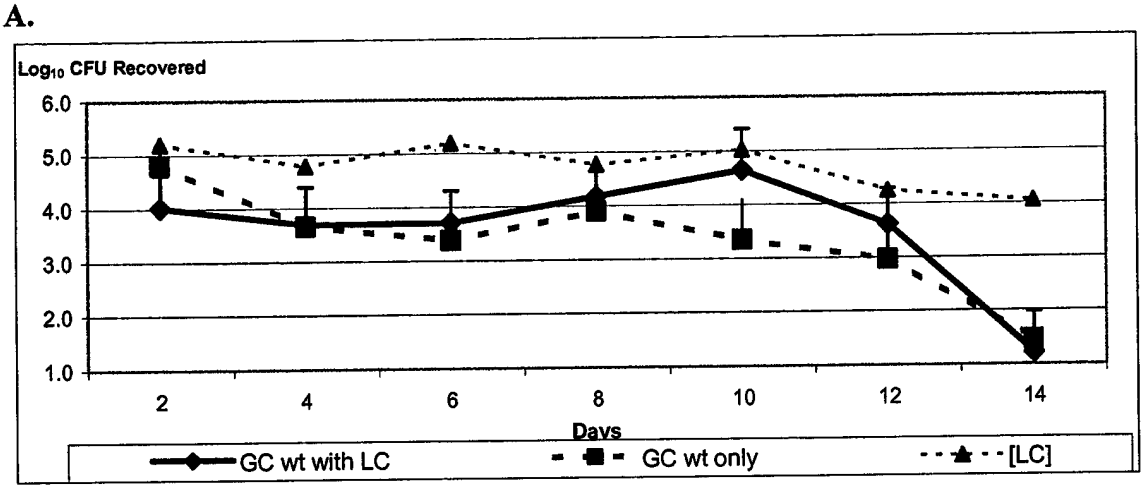
Vaginal smears were reviewed to determine if inflammation occurred during infection. In all situations in which mice cleared bacterial infection, an influx of PMNs was detected above baseline. This result suggests that clearance of both wild-type and catalase mutant was associated with host inflammatory response and not the presence of lactobacilli.

Table 6. Average duration of recovery of *N. gonorrhoeae* FA1090 versus *N. gonorrhoeae* 24.2 (catalase mutant) from mice in the presence or absence of *L. crispatus*.

	Duration Recovery of *N. gonorrhoeae* Days (standard error) (range)
L. crispatus + GC FA1090	12.3 (0.24) (8-14)
GC FA1090	12.5 (0.29) (2-14)
L. crispatus + GC 24.2	12.8 (0.24) (6-14)
GC 24.2	11.8 (0.30) (4-14)

Figure 12. Average recovery of *N. gonorrhoeae* FA1090 (A) and 24.2 (B) from mice in the presence or absence of *L. crispatus* following challenge with 1.8×10^6 CFU (FA1090) and 4.2×10^5 CFU (24.2). For comparative purposes, panel C depicts the results for both gonococcal strains. Results are expressed as Log_{10}CFU recovered per 100 µl vaginal swab suspension. Limit of detection: 4 CFU/100 µl vaginal swab suspension.

A.



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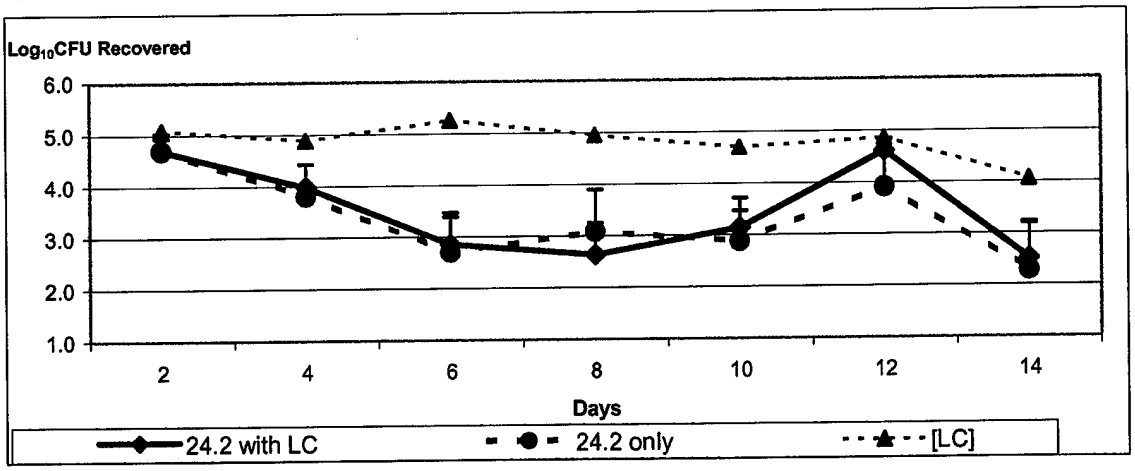

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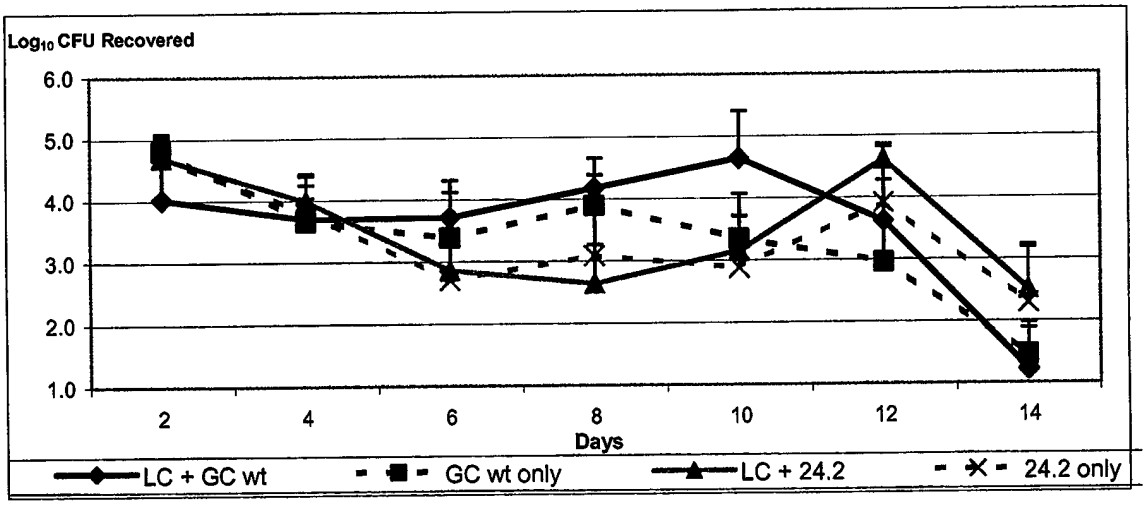
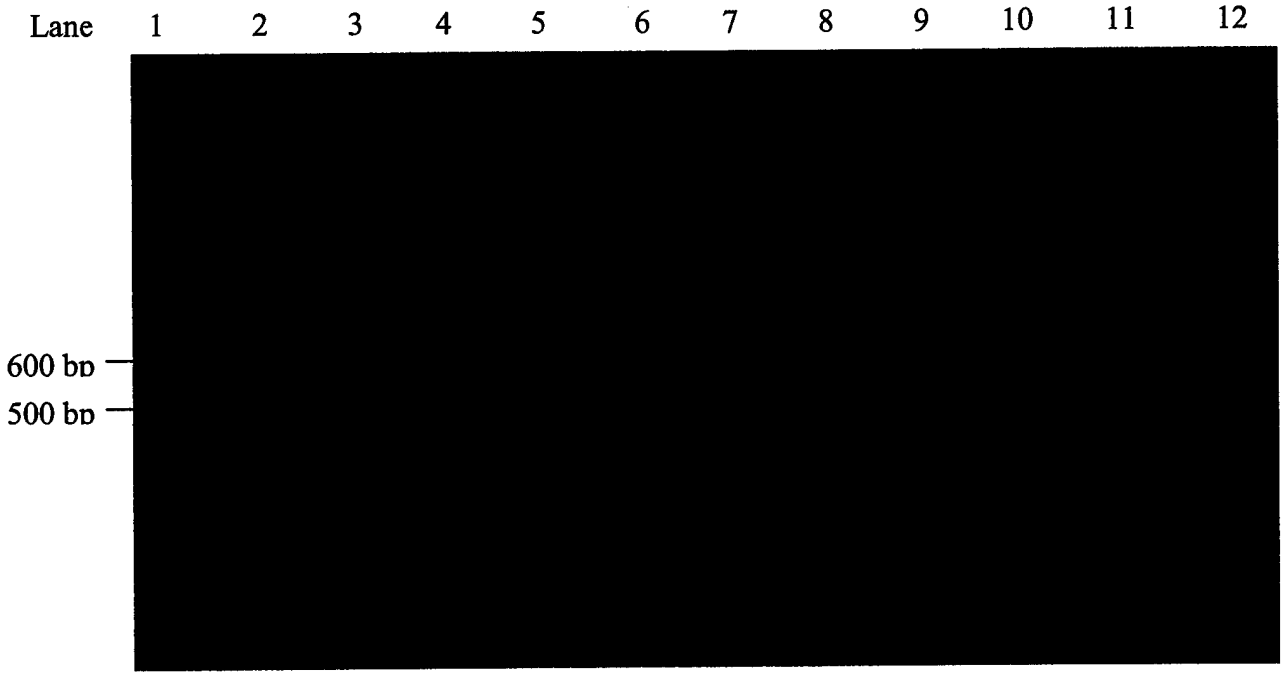


Figure 13. PCR identification of presumptive *L. crispatus* isolates from the lower genital tracts of mice during protection experiments. Lanes 1 and 3 contain a 100-bp DNA ladder. Lane 2 contains *L. crispatus* (positive control), and lanes 4 through 12 contain *L. crispatus* isolates from mouse vaginal cultures (PCR-product 522-bp).



b. ***Experiment 2.*** Results from the first protection experiment showed no evidence of *L. crispatus* reducing the duration of recovery or bacterial load of wild-type or catalase-deficient *N. gonorrhoeae* at a challenge dose of 1 x 10^6 and 4 x 10^5 CFU respectively. To repeat this experiment, we chose to challenge the mice that were inoculated with approximately 10^7 lactobacillus CFU or placebo control with the lower dose (5 x 10^5 CFU wild-type or catalase mutant). Again, no significant difference in the duration of *N. gonorrhoeae* recovery was detected between the four mouse test groups (Table 7). The average recovery of *N. gonorrhoeae* as a function of time in mice pre-colonized with *L. crispatus* versus the control group was also evaluated. As shown in Figure 14A, there was no statistically significant difference in recovery of *N. gonorrhoeae* FA1090 from mice with *L. crispatus* and without *L. crispatus*. There was also no significant difference in the degree of recovery (bacterial load) of the catalase-deficient mutant (Figure 14B).

As before, *L. crispatus* was recovered throughout the experiment. The recovery of *L. crispatus* from the respective mouse groups is shown on Figures 14A and 14B (dotted line). The identity of lactobacillus vaginal isolates was confirmed by PCR-analysis (data not shown). The recovery of the respective gonococcal strain from all four treatment groups is compared in figure 14C. The recovery of FA1090 from mice with and without *L. crispatus* colonization, and *N. gonorrhoeae* 24.2 from mice not colonized with *L. crispatus* was comparable throughout the course of the experiment. However, in this experiment, although not statistically significant, the recovery of *N. gonorrhoeae* 24.2 from mice colonized with *L. crispatus* was lower than the number of gonococci recovered from the other three treatment groups. This observation suggests that gonococcal catalase may protect against hydrogen peroxide produced by lactobacilli.

Future experiments with a lower challenge dose (i.e., 1 x 10^5 gonococci CFU) may further elucidate this theory.

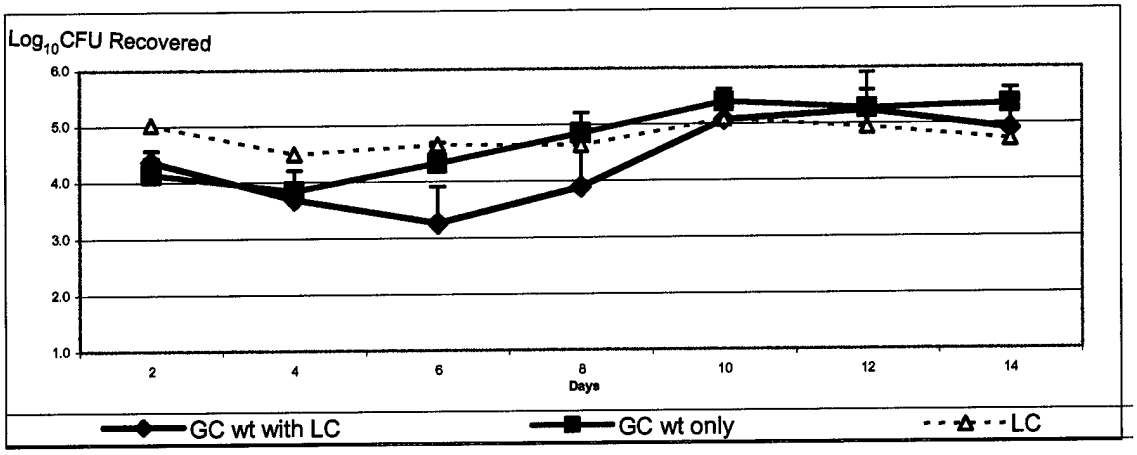
In both experiment 1 and 2, an interesting observation was made regarding recovery of lactobacilli from the murine vaginal tract (dotted lines in Figures 12A-B and 14A-B). Although these mice received > 5 x 10^6 *L. crispatus* CFU/100 µl vaginal swab suspension during initial and booster inoculations, the number of CFU recovered from the mice following these inoculations ranged from approximately 10^4 to 10^5 CFU/100 µl vaginal swab suspension. This observation suggests that lactobacillus colonization is a function of receptor availability, biofilm dynamics, and/or another unknown restrictive mechanism such as clearance by innate defenses. Lactobacilli that did not colonize the murine vaginal tract were assumed to be removed by the flushing action of the vaginal tract.

Table 7. Average duration of recovery of *N. gonorrhoeae* FA1090 versus *N. gonorrhoeae* 24.2 (catalase mutant) from mice in the presence and absence of *L. crispatus*.

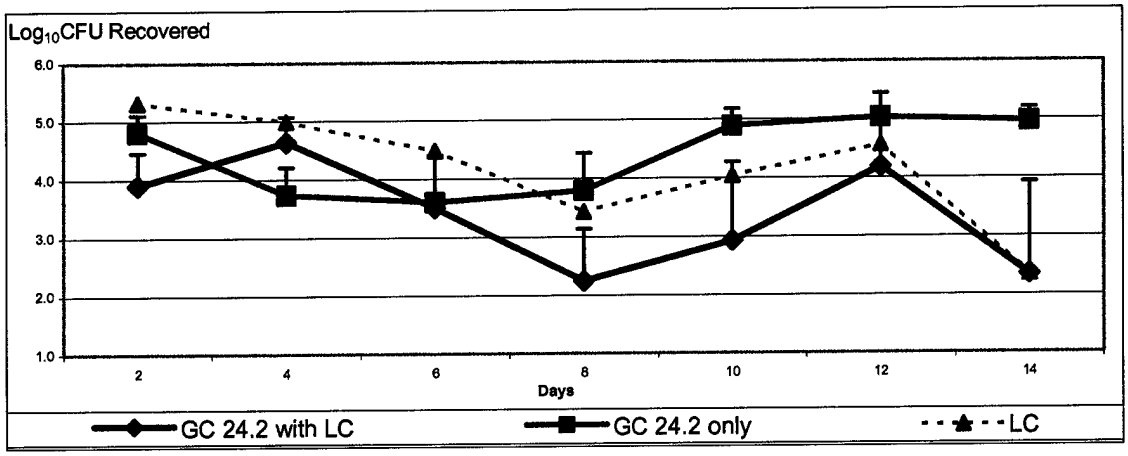
	Duration Recovery of *N. gonorrhoeae* days (standard error) (range)
L. crispatus + GC FA1090	13.5 (0.2) (10-14)
GC FA1090	14.0 (0) (14)
L. crispatus + GC 24.2	13.0 (0.4) (12-14)
GC 24.2	14.0 (0) (14)

Figure 14. Average recovery (log_{10} CFU/100 µl) of *N. gonorrhoeae* FA1090 (A) and *N. gonorrhoeae* 24.2 (B) from mice in the presence or absence of *L. crispatus* following challenge with 4.9 x 10^5 CFU (FA1090) and 6.4 x 10^5 CFU (24.2). For comparative purposes, results for both strains are depicted in C. Results are expressed as Log_{10}CFU recovered per 100 µl vaginal swab suspension. Limit of detection: 4 CFU/100 µl vaginal swab suspension.

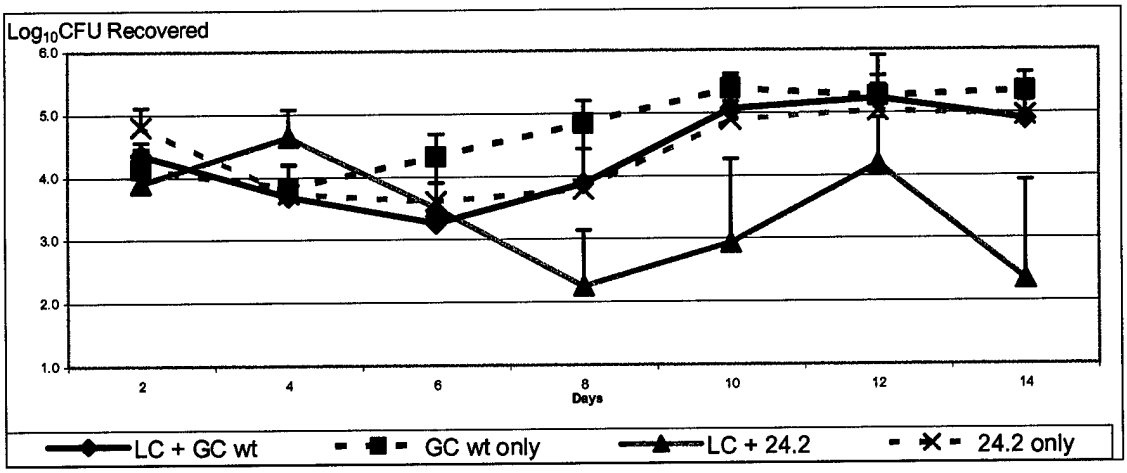
A.



B.



C.



c. ***Murine Vaginal pH during in vivo protection studies.*** The average mucosal pH of the murine lower genital tract during our in vivo protection studies is presented in Table 8. As observed in the mouse lactobacillus colonization studies, the presence of lactobacilli did not seem to have a major effect on the vaginal acidity of the mice. Additionally, the data indirectly suggest that the presence of *N. gonorrhoeae* did not induce an increase in lactate production by *L. crispatus*.

Table 8. Vaginal pH of murine lower genital tract during lactobacillus protection experiments 1 and 2.

	Average Mouse Vaginal pH (Standard Error)		
Experiment 1	**Day 4**	**Day 8**	**Day 12**
LC + GC FA1090	6.45 (0.17)	6.58 (0.05)	6.85 (0.08)
GC FA1090 only	6.75 (0.19)	6.53 (0.10)	6.74 (0.14)
LC + GC 24.2	6.52 (0.17)	6.59 (0.06)	6.64 (0.13)
GC 24.2 only	6.64 (0.18)	6.6 (0.09)	6.88 (0.06)
Experiment 2	**Day 4**	**Day 8**	**Day 12**
LC + GC FA1090	6.68 (0.16)	6.77 (0.15)	6.49 (0.12)
GC FA1090 only	6.72 (0.15)	6.47 (0.19)	6.40 (0.09)
LC + GC 24.2	6.84 (0.19)	6.85 (0.13)	6.86 (0.12)
GC 24.2 only	6.33 (0.16)	6.51 (0.16)	6.50 (0.14)

DISCUSSION

For the last twenty years, hydrogen peroxide-producing *Lactobacillus spp.* have been hypothesized to protect against gonorrhea. We tested this hypothesis with *in vitro* studies and *in vivo* experimentation using the murine *N. gonorrhoeae* genital tract infection model.

I. *In vitro* inhibition of *N. gonorrhoeae* by human vaginal hydrogen peroxide-producing *Lactobacillus spp.* and selection of *L. crispatus* (ATCC 33197) as the best candidate probiotic strain. The first objective was to define and/or characterize the inhibition of *N. gonorrhoeae* by two strains that represent the two *Lactobacillus spp.* that predominant in the female lower genital tract, namely *L. crispatus* and *L. jensenii*. Data from gel overlay assays showed that the primary mechanism of *in vitro* inhibition of *N. gonorrhoeae* by *L. crispatus* and *L. jensenii* is the production of hydrogen peroxide. This conclusion was derived from the following observations. First, lactobacilli that lack the ability to produce hydrogen peroxide failed to inhibit *N. gonorrhoeae*. Additionally, inhibition of *N. gonorrhoeae* by hydrogen peroxide-producing strains was neutralized by the addition of exogenous catalase. In fact, more exogenous catalase was required to neutralize the inhibition by *L. crispatus* as compared to *L. jensenii*, a result that is consistent with the greater inhibition of gonococci by *L. crispatus*. This result is also consistent with the report that *L. crispatus* produces the highest concentrations of hydrogen peroxide among *Lactobacillus spp.* (*Ocana et al*, 1999a). We presume the hydrogen peroxide responsible for the inhibition is produced prior to the application of the overlay media, which probably creates an anaerobic environment for the lactobacilli and thus, prevents further production of hydrogen peroxide. We hypothesize that the

hydrogen peroxide produced prior to addition of the overlay diffuses through the overlay media and inhibits the growth of the gonococci. In strong support of this hypothesis is the demonstration that addition of bovine catalase to the overlay media neutralizes *Lactobacillus* inhibition of *N. gonorrhoeae*. The *in vitro* inhibition was bactericidal, as culturing within the zone of gonococcal growth inhibition failed to produce viable gonococci. Bactericidal activity is consistent with the ability of hydrogen peroxide to disrupt both molecular and cellular processes in bacteria.

The contribution of catalase in the protection of *N. gonorrhoeae* from hydrogen peroxide-producing lactobacilli was also revealed by gel overlay experiments. When *L. crispatus* and *L. jensenii* were co-incubated with a mutant strain of *N. gonorrhoeae* in which the ability to produce catalase was genetically abolished, the catalase mutant was more sensitive than the wild-type parent to lactobacillus inhibition. As predicted, more exogenous catalase was required to neutralize the inhibition of the catalase-deficient mutant by hydrogen peroxide-producing lactobacilli.

The increased inhibition of *N. gonorrhoeae* by *L. crispatus* under acidic conditions supports the theory that acidity stabilizes hydrogen peroxide. *Fontaine and Taylor-Robinson* (1990) observed a 75% decrease in hydrogen peroxide stability at neutral/alkaline pH, and showed acidity is essential for the stability of hydrogen peroxide. *Aroutchera et al* (2001) found the activity of lactobacilli-produced hydrogen peroxide to be dependent on pH. *Zheng et al* (1994) also observed an increase in lactobacillus inhibition of gonococci at acidic pH; however, these investigators attributed the inhibitory activity to the production of a bacteriocin by lactobacillus at low pH. We cannot rule out the possibility that acidic conditions increase production of other

inhibitory factors. However, because bovine catalase neutralized the inhibition at lower pH, the possibility of increased production of hydrogen peroxide at low pH cannot be excluded.

Although *in vitro* inhibition studies showed *L. crispatus* inhibited *N. gonorrhoeae* more than *L. jensenii* many factors must be considered when selecting a bacterium to serve as a probiotic. Therefore, the second objective of our research was to investigate the other factors that might promote the success of lactobacillus-induced inhibition of gonococci. When considering vaginal lactobacilli, important aspects to consider are colonization capability and the ability to acidify the immediate environment. Consequently, a more complete understanding of the effect of lactobacilli on the murine genital tract was required in order to design and interpret subsequent *in vivo* lactobacilli protection studies.

We first looked at the ability of each strain to colonize mice. Despite the fact that *L. crispatus* and *L. jensenii* were isolated from humans, we successfully colonized mice with streptomycin-resistant strains of *L. crispatus* and *L. jensenii*. In pilot studies, a decline in the number of lactobacilli occurred at day 5. Consequently, *in vivo* protection studies were designed to include a *L. crispatus* "booster" that was administered to the mice to maintain the highest possible concentration of lactobacilli. Boosting the mice on day 5 and day 10 helped to maintain high levels of lactobacilli colonization over the course of the experiment. We also assessed the adherence of *L. crispatus*, *L. jensenii*, and *L. murinus* to vaginal epithelial cells *in vivo*. All three strains showed equal degrees of association with epithelial cells in stains smears, with some evidence of coaggregation.

We then looked at acidification *in vitro*. *L. crispatus*, *L. jensenii*, and *L. murinus* all acidified the culture media over time. In the case of *L. crispatus* and *L. murinus*, acidity was associated with the production of lactate. *L. jensenii* also acidified the growth media, but the delayed detection of lactate with respect to the time at which pH began to decrease suggested that the cause of this acidification was not solely lactate production. Perhaps *L. jensenii* is one of the lactic acid bacteria that utilizes the heterofermentative mechanism of metabolism, and thus produces acidic fermentative by-products such as acetic acid, formate, and succinate in addition to lactate.

In pilot experiments, colonization of estradiol-treated mice with *L. crispatus, L. jensenii,* or *L. murinus* did not dramatically decrease the vaginal pH as expected. As discussed in the Introduction, human vaginal acidification is dependent on substrate availability (glucose from glycogen) and the number of lactobacilli present. After many failed attempts to achieve vaginal acidification in our model, we pondered the following explanations. We assume glucose is present as a result of high estrogen levels and epithelial "sloughing"; future measurements of glucose in vaginal washings should easily resolve this issue. Pooled vaginal swab suspensions did reveal that lactate was present in the vaginas of all the mouse groups (data not shown). Alternatively, one other possibility is that mice may possess a unique vaginal buffering system, which maintains a neutral pH. Finally, the inability to colonize the mouse vagina with the levels of lactobacilli needed to produce a "truly" acidic pH could also explain the results. Antonio et al (1999) estimated 1 x 10^8 lactobacilli/ml was required to produce acidic conditions. In our hands, inoculation of mice with 10^9 lactobacillus CFU typically yielded 10^4 to 10^5 CFU/100 μL vaginal swab suspension per day. We believe this is the highest level of colonization that

we can achieve; it should be noted that *L. murinus*, a mouse commensal, did not colonize mice at higher numbers following inoculation with 10^9 CFU. This observation can be explained by the limited number of vaginal epithelial cell receptors used by lactobacillus, and/or the presence of conditions that were not conducive to *L. murinus* colonization.

In summary, *L. crispatus* inhibited *N. gonorrhoeae* more than *L. jensenii in vitro* and produced lactate earlier during *in vitro* culture. All other parameters were the same. Based on these results, *L. crispatus* was selected as the best probiotic candidate and thus utilized for further study.

II. Absence of *in vivo* inhibition of *N. gonorrhoeae* by human vaginal hydrogen peroxide-producing *Lactobacillus spp.* The work described here is the first attempt to study the interaction of hydrogen peroxide-producing lactobacilli with *N. gonorrhoeae* in an *in vivo* infection model. Although the use of an animal model for the study of a human-specific pathogen has limitations, the murine model was the best available system to test our hypothesis. To our surprise, the results did not support our hypothesis that *L. crispatus* would prevent or attenuate gonococcal infection in mice. Colonization of mice with *L. crispatus* did not reduce the recovery of wild-type or a catalase-deficient gonococci. Protection studies utilizing *L. jensenii* (data not shown) also failed to protect mice from the colonization of *N. gonorrhoeae in vivo* (data not shown). We have considered many possible explanations for the inability of *L. crispatus* to protect the murine lower genital tract from gonococcal challenge.

a. ***Hydrogen peroxide may not be produced by L. crispatus in vivo.*** Although all the *L. crispatus* isolates from mouse vaginal cultures produced hydrogen peroxide when

assessed two passages after isolation, it is not clear if *L. crispatus* produced hydrogen peroxide *in vivo*. As already discussed, oxygen is required by lactobacilli to produce hydrogen peroxide. Therefore, *in vivo* hydrogen peroxide production by lactobacilli is dependent on vaginal oxygen tension. Under normal conditions, the vaginal lumen is considered hypoxic in that it has low to near-zero partial pressure of oxygen (*Eskow and Loesche*, 1971; *Wagner and Levin*, 1978). This level is not conducive to hydrogen peroxide production by lactobacilli. However, human studies have shown that the vaginal partial pressure of oxygen can be increased dramatically by sexual arousal (*Wagner and Levin*, 1978), insertion of female hygiene products (e.g., tampons) (*Wagner et al*, 1984), and/or insertion of contraception devices (e.g., diaphragm) (*Wagner et al*, 1988). The elevated oxygen tension that resulted from such manipulation gradually declined over the course of hours, but specific elevation and dissipation rates were dependent on the method and amount of oxygen introduced into the lumen. Consistent with these human studies, we believe the repeated insertion of the pH microelectrode and the cotton-culturing swab may have introduced a sufficient amount of molecular oxygen to the normally anaerobic vaginal lumen for the facultative lactobacilli to be able to produce hydrogen peroxide. Unfortunately, the lack of an effective method to measure *in vivo* hydrogen peroxide concentrations prevented us from testing this assumption. Therefore, the absence, or a reduced rate of hydrogen peroxide production *in vivo* could have resulted in the observed lack of *in vivo* inhibition of gonococci by lactobacilli.

b. ***Lactobacillus acidity and/or hydrogen peroxide-production may be reduced due to biofilm formation in the murine vaginal tract.*** Many microorganisms, including *Lactobacillus spp.*, are known to exist as biofilms in both natural and artificial

environments (*Sutherland*, 2001). Cytological examination of murine vaginal smears during our *in vivo* lactobacillus colonization studies revealed the presence of lactobacilli in "sheets/films," an observation suggestive of *in vivo* biofilm formation. The presence of these bacterial films became more apparent 4 days after initial colonization.

The formation of biofilms creates a complex and dynamic environment that can result in nutrient limitation, and/or altered bacterial metabolic activity (*Fenchel*, 2002; *Gilbert et al*, 2002). The competition between large numbers of lactobacilli in close proximity to one another can limit the availability of glucose and molecular oxygen, which is of importance to the interpretation of our *in vivo* results. First, glucose limitation could result in decreased lactate production by lactobacilli, and ultimately contribute to the lack of vaginal acidity. Additionally, reduction of the already low levels of oxygen through bacterial competition and/or limited diffusion within the biofilm matrix could limit the formation of hydrogen peroxide, which is believed to be the primary inhibitory mechanism utilized by lactobacilli against *N. gonorrhoeae*. Yet other factor to be considered is the potentially reduced metabolic rate of the lactobacilli within a biofilm. Reduction in lactobacillus metabolism could result in reduced lactate formation and/or hydrogen peroxide production. As described earlier, both vaginal acidity (via lactate production) and hydrogen peroxide are believed to be necessary for inhibition of *N. gonorrhoeae*.

Experimental inoculation of high numbers of *L. crispatus* in the murine vagina may have inadvertently initiated the formation of biofilms. High concentrations of *L. crispatus* (ranging from 5×10^6 to 1×10^7 CFU) were used to inoculate and boost mice during *in vivo* protection studies to maintain approximately 10^5 *L. crispatus* CFU per

vaginal swab suspension over the course of the experiment. Conversely, natural colonization of mice with *L. murinus* was observed to not exceed 10^3 CFU/100 µl vaginal swab suspension (Figure 9). Consequently, inoculating the murine vaginal tract with high levels *of L. crispatus* may have forced biofilm formation, and subsequently resulted in the inability of *L. crispatus* to inhibit *N. gonorrhoeae* during our *in vivo* protection studies.

c. ***Hydrogen peroxide produced by L. crispatus may be unstable at neutral vaginal pH.*** If hydrogen peroxide is indeed produced *in vivo* by lactobacilli, an acidic environment is needed to stabilize the oxidant's presence (*Fontaine and Taylor-Robinson,* 1990; *Aroutchera et al,* 2001). Consequently, the instability of hydrogen peroxide in the relatively neutral murine vaginal tract may have contributed to the lack of gonococcal inhibition.

d. ***Hydrogen peroxide produced by L. crispatus may be neutralized by lactobacillus-derived catalase.*** To ensure hydrogen peroxide produced by lactobacilli was not being destroyed by lactobacillus-derived catalase, the ability of *L. crispatus* to produce catalase was assessed. The *L. crispatus* strain studied did not possess the ability to produce catalase when tested using the bubbling assay (data not shown). *L. crispatus* and *L. jensenii* must possess an alternative anti-oxidative mechanism to allow survival in the presence of hydrogen peroxide produced by the lactobacilli.

e. ***Alternative anti-oxidative mechanisms utilized by N. gonorrhoeae.*** Another theory that must be addressed is that *N. gonorrhoeae* may utilize an alternative anti-oxidative mechanism to combat hydrogen peroxide that is induced *in vivo,* but is not produced or effective in gel overlay assays. This theory could explain the lack of

attenuation of both the wild-type and catalase-deficient mutant *N. gonorrhoeae* strains during murine infection in the presence of hydrogen peroxide-producing lactobacilli.

Microorganisms have developed several strategies to avoid the oxidative stress conferred by reactive oxygen species. These strategies include repair of damaged DNA, formation/expression of new proteins, and production of antioxidative enzymes (*Morgan et al*, 1986). The bacterially derived enzymes superoxide dismutase (SOD), glutathione sulfhydroreductase (GSH), peroxidases, and catalase are employed to deactivate oxidants encountered in the environment, and to convert toxic reactive oxygen species to nontoxic end products. The recovery of *N. gonorroheae* from neutrophil-laden environments, which presumably contain high levels of hydrogen peroxide as part of the PMN defense, suggests gonococci avoid damage from hydrogen peroxide. Gonococci produce little to no superoxide dismutase SOD (*Norrod and Morse*, 1979), and only a few gonococci possess the ability to produce GSH (*Archibald and Duong*, 1986). Consequently, the major known gonococcal antioxidative mechanism is the production of catalase and/or peroxidase (*Archibald and Duong*, 1986).

The conditions in which *N. gonorrhoeae* resides, and the factors encountered, may directly affect sensitivity to hydrogen peroxide. These conditions and factors can be both host and bacterially derived. Studies by Alcorn et al (1994) suggest gonococcal catalase is just one of many factors that determine gonococcal sensitivity to hydrogen peroxide. This study showed resistance to hydrogen peroxide was strain specific, suggesting other factors within gonococci affect sensitivity to exogenous hydrogen peroxide. Archibald and Duong (1986) hypothesized that the presence of intracellular bacterial GSH potentially affects gonococcal sensitivity to hydrogen peroxide. Cohen et

al (1987) observed the presence of host-derived iron affected the susceptibility of gonococci to redox stress. Finally, the concentration and reducing capabilities of endogenous reactive oxygen species are yet another factor to consider in studying gonococcal sensitivity to hydrogen peroxide.

In the absence of the ability to produce catalase, enzymes such as peroxidase and GSH can be potentially utilized by gonococci to degrade hydrogen peroxide. Archibald and Duong (1986) found that gonococci constitutively produce high levels of peroxidase. Peroxidase production could be up regulated *in vivo*, thereby affording protection to the catalase-deficient mutant. GSH can catalyze the destruction of hydrogen peroxide in the presence of glutathione peroxidase and glutathione reductase (*Haas and Goebel*, 1992). During the reaction, GSH serves as a substrate acted on by the enzyme glutathione peroxidase, and then catalyzes the degradation of hydrogen peroxide. GSH activity has been demonstrated in many bacterial genera (*Piccolomini et al*, 1989). Of note, a GSH peroxidase homolog (*gpxA*) was identified in *N. meningitidis (Moore and Sparling,* 1995). A *gpxA* homolog has not been yet identified in *N. gonorrhoeae*, however, perhaps gonococci possess GSH-metabolizing proteins, which would explain the detection of GSH-activity by Archibald and Duong (1986). The completion of the FA1090 genome sequence will help to elucidate the antioxidative enzymes possessed by FA1090, the strain utilized in our studies. Glutathione peroxidase and reductase may not be present in the gel overlay assay, which may account for the observed *in vitro* inhibition of *N. gonorrhoeae* by hydrogen peroxide-producing lactobacilli.

A non-enzymatic mechanism of anti-oxidation is the intracellular accumulation of manganese (2+). This mechanism has been most thoroughly characterized in *L.*

plantarum, where intracellular manganese (2+) was shown to serve as a biological auto-oxidant (*Archibald and Duong*, 1981). *Tseng et al* (2001) observed gonococcal resistance to oxidative killing, and attributed this protection to manganese (2+) uptake and accumulation based on the increased sensitivity of a manganese uptake mutant to oxidative stress. The presence or increased presence of manganese (2+) *in vivo* could therefore account for the lack of lactobacillus protection against gonococci during experimental murine infection.

Yet another viable explanation for the lack of inhibition of gonococcal infection by *L. crispatus* is the presence of a yet uncharacterized inducible antioxidant mechanism. *Fu et al* (1989) found that *N. gonorrhoeae* display a change in phenotype upon oxidative stress, which is marked by an increase in lactate dehydrogenase (LDH) activity, resulting in the utilization of L-(+)-lactate. In this study, oxidatively "stressed" gonococci utilized L-(+)-lactate more readily than other carbon sources coupled with an increase in LDH activity. It is not clear how LDH plays a role in adaptation to oxidative stress. However, if utilization of lactate is important, the presence of lactobacillus-derived lactate would serve as a huge benefit to *N. gonorrhoeae*, and help explain the lack of inhibition observed in the *in vivo* protection studies.

Finally, the gonococcal iron storage protein, Bfr, has been recently shown to play an important role in the protection of gonococci against oxidative stress (*Chen and Morse*, 1999). In these studies, *bfr*-mutant gonococci were more sensitive to hydrogen peroxide. Gonococci are iron-stressed during experimental murine infection (*Jerse et al*, 2002), an observation that lends support to the likelihood that the expression of *bfr* is increased. If expression of *bfr* is indeed increased during experimental murine infection,

this could explain the reason that gonococci appeared to be protected against hydrogen peroxide produced by lactobacilli *in vivo*.

f. ***Inhibition observed in clinical studies may be the cumulative effect of many commensal bacteria.*** A plausible explanation for the lack of *in vivo* inhibition is that more than one bacterial genus or species is responsible for the reduced risk of gonorrhea observed in women colonized by hydrogen peroxide-producing lactobacilli. Inhibition of *N. gonorrhoeae* by commensal flora in women may be a synergistic relationship among a community of lower genital tract bacteria that together create an environment that inhibits gonococcal infection. As discussed in the Introduction, healthy women are normally colonized with several species of lactobacillus, both hydrogen peroxide and non-hydrogen peroxide producers, as well as other genera. To test this hypothesis one could colonize mice with a lactobacillus population more representative of the population present in the healthy human vaginal and endocervix. This lactobacillus population would include a variety of strains with varying abilities to produce hydrogen peroxide, lactate, bacteriocins, and biosurfactants. This design would be more representative of the conditions in women described in clinical correlation studies

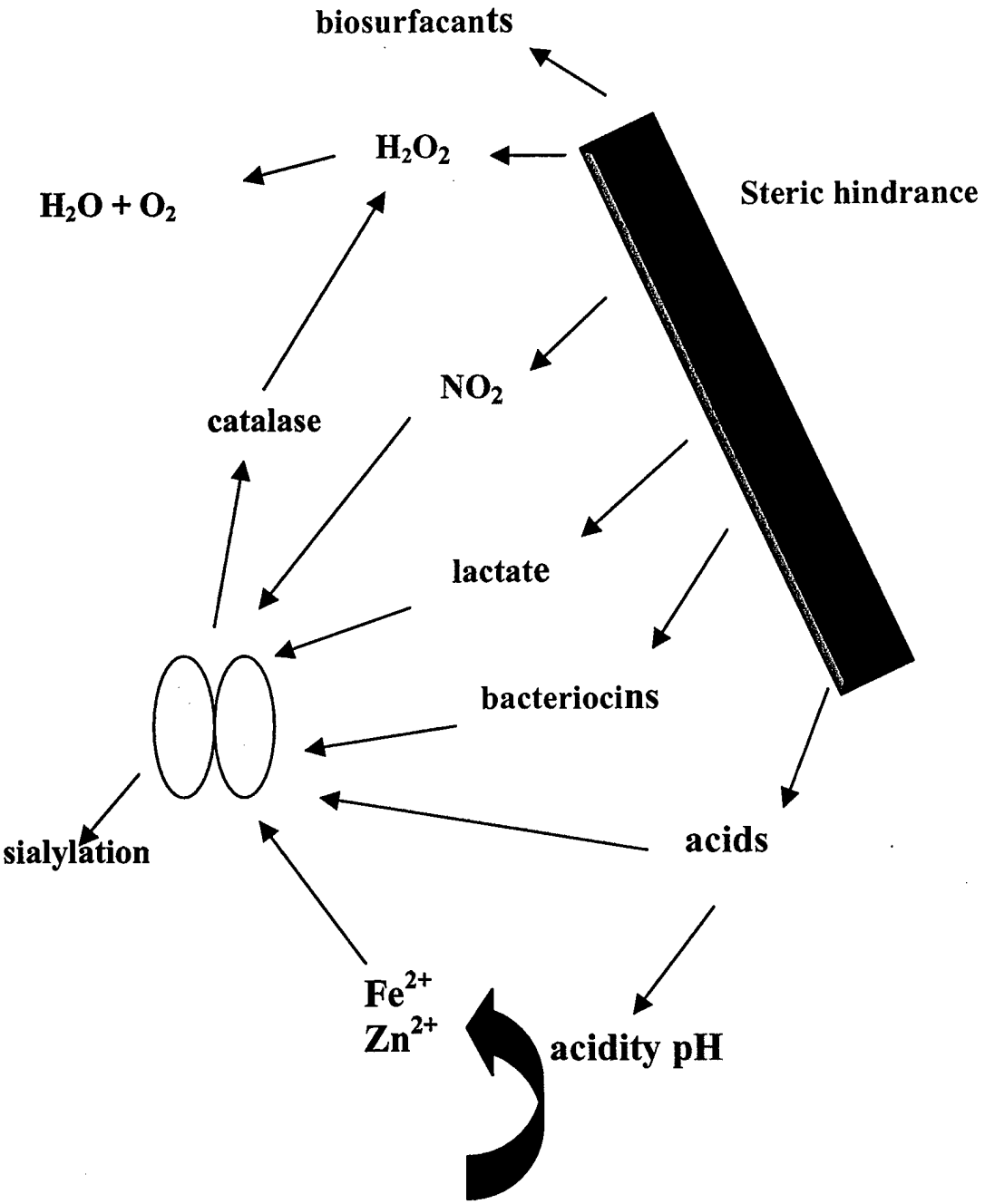
III. Complicated interaction between *Lactobacillus spp.* and *N. gonorrhoeae*. The interaction between lactobacilli and gonococci may be more complicated than originally theorized. Unfortunately, due to the complex nature of the female lower genital tract, the components that compose this ecosystem are difficult to study independently. Lactobacilli produce many by-products and conditions that can either enhance or inhibit *N. gonorrhoeae*. Such interactions might generate a complicated relationship that can be thought of as a balance between increased survival and/or enhancement, and inhibition.

Some of the potential interactions between lactobacilli and *N. gonorrhoeae* are outlined in Figure 15.

a. ***Potential inhibitory conditions generated by Lactobacillus spp.*** Although the focus of my research is the inhibition of *N. gonorrhoeae* by *Lactobacillus spp.* via hydrogen peroxide production, many other potential inhibitory mechanisms must be considered when studying the interaction between lactobacilli and gonococci. Theoretically, lactobacilli can also inhibit *N. gonorrhoeae* by competitive exclusion, coaggregation, and the production of bacteriocins and/or biosurfactants.

The presence of lactobacilli on epithelial cell surfaces may block the adherence of pathogens, and thus prevent colonization. This inhibitory mechanism may occur by competitive exclusion of the pathogen from epithelial surface receptors (*Chan et al*, 1985), and/or by steric hindrance (*Reid and Sobel*, 1987). Although the exact molecular adherence mechanism utilized by lactobacilli still remains unclear, it is believed that charge interactions are involved in the initial attraction, followed by adhesion mediated by proteins (*Conway and Kjellberg*, 1989; *Henricksson et al*, 1991), lipoteichoic acid (*Chan et al*, 1985), or carbohydrate (*Brooker and Fuller*, 1975; *Fuller*, 1975). *In vitro* studies demonstrated that cell wall fragments from *Lactobacillus spp.* blocked pathogen adherence to urogenital epithelial cells (*Reid and Sobel*, 1987; *Chan et al*, 1985). Boris et al (1998) revealed lactobacilli compete for epithelial cell surface receptors with urogenital pathogens *Gardnerella vaginalis* and *Candida albicans*. Further investigation revealed lactobacilli had a much higher affinity for the surface receptors, and could displace pathogens already bound to the cell surface. Interestingly, pH was not found to be a factor in adhesion of lactobacilli to vaginal epithelium (*Ocana et al*, 2001).

Figure 15. Potential interactions between *Lactobacillus spp.* (depicted as purple bacillus) and *N. gonorrhoeae* (depicted as pink diplococci) *in vivo*. By-products and conditions depicted in green are considered enhancing, while by-products and conditions shown in red are considered inhibitory.



The ability of lactobacilli to coaggregate with other bacteria may also inhibit successful colonization of genital pathogens by impeding the pathogen's acess to tissue receptors. *Lactobacillus spp.* can coaggregate in vaginal (*Reid et al*, 1988 and 1990) and oral cavities (*Chan et al*, 1984 and 1985). Vaginally isolated lactobacilli have the ability to coaggregate *in vitro*, and consequently are hypothesized to impede the pathogen's access to tissue receptors. Specifically, *in vitro* studies observed vaginal lactobacillus isolates coaggregated *Candida albicans, Gardnerella vaginalis,* and *Escherichia coli* (*Boris et al*, 1998). Interestingly, this same study reported that coaggregates of *Streptococcus agalactiae* and vaginal lactobacilli did not form, a result that suggests the process of coaggregation is specific. The ability of lactobacilli to coaggregate within the lower genital tract is vital for forming complex microbial communities within biofilms. Additionally, coaggregation may indirectly enhance inhibition of bacteria when coupled with the production of hydrogen peroxide, bacteriocins, and other organic compounds.

Many lactic acid bacteria produce bacteriocidal proteins called bacteriocins possessing broad antibacterial activity (*Jack et al*, 1995). Vincent et al (1959) first described the "bacteriocin-type" inhibition of *Proteus spp., Staphylococcus spp., Bacillus spp., Streptococcus spp., and Lactobacillus spp.* by *L. acidophilus*. This inhibitory agent, a bacteriocin, was later identified as lactacin B (*Barefoot and Klaenhammer*, 1983). Many lactic acid bacteria produce bacteriocins, which are defined as bacteriocidal proteins produced by bacteria (*Jack et al*, 1995). The plasmid-mediated bacteriocins of Gram positive bacteria possess membrane activity through the formation of ion-channels that disrupt cell function (*Bruno and Montville*, 1993; *Christensen and Hutkins*, 1992). Kanatani et al (1995) later identified acidocin A produced by *L. acidophilus*. Ocana et al

(1999; reference 99) identified and characterized a bacteriocin produced by vaginal *L. salivaris subspecies salivarius*. This protein was shown to have inhibitory activity against *Neiserria gonorrhoeae*. *L. sakei*, another vaginal *Lactobacillus spp.*, has also been found to produce a bacteriocin (*Leroy and Vuyst*, 2001).

Biosurfactants (i.e., glycolipids, lipopeptides, phospholipids) released by lactobacilli accumulate at liquid-air interfaces where they can play an anti-colonization role. Biosurfactants produced by lactobacilli can also have an important anti-microbial function (*van der Vegt et al*, 1991). Velraeds et al (1996) found all lactobacilli produce biosurfactants at varying rates, and that lactobacillus isolates with high rates of biosurfactant production inhibited adhesion of *Enterococcus faecalis in vitro*. Later, this research team found that the *L. acidophilus* biosurfactant surlactin interfered with the adhesion of numerous uropathogens (*Velraeds et al*, 1998).

b. ***Potential enhancement conditions for N. gonorrhoeae generated by Lactobacillus spp.*** Although not fully characterized, lactobacilli produce many compounds that may enhance the growth and survival of gonococci, namely lactate, nitrite, and acidity. *In vitro* and *in vivo* experiments conducted in our laboratory have shown that non-hydrogen peroxide-producing *L. murinus* strains enhanced the growth of gonococci unless an iron chelator is added to the media. This result suggests that *L.murinus* creates a more iron rich environment, possibly by producing metabolites that can chelate iron to increase iron availability to *N. gonorrhoeae* (*Jerse et al*, 2002). Interestingly, we routinely recover high numbers of gonococci from mice colonized with *L. murinus*, suggesting this organism creates a hospitable environment *in vivo* as well.

Lactate can serve as a growth substrate for *N. gonorrhoeae in vitro* (*Barron and Hastings*, 1933). Gonococci possess three known lactate dehydrogenase (LDH) enzymes, which allow the bacteria to oxidize lactate to pyruvate. Numerous studies have provided evidence that lactate stimulates the metabolism of *N. gonorrhoeae*, and that gonococcal sialyltransferase activity is increased when grown with lactate, thereby increasing sialylation of its LOS. Sialylated gonococci are more resistant to complement-mediated killing and opsonophagocytosis, and therefore, the presence of high concentrations of lactate due to lactobacilli may promote evasion of the host innate immune system (*Smith et al,* 2001).

Finally, strains of lactobacilli have also been found to produce nitrite (*Lin and Lai*, 1982). This nitrite, when available, can serve as a terminal electron acceptor to allow gonococci to grow anaerobically (*Knapp and Clark*, 1984). This anaerobic growth may be an advantage in the genital tract, where the partial pressure of oxygen is low.

IV. Conclusions. The intent of this research was to gain a better understanding of the interaction between human vaginal hydrogen peroxide-producing lactobacilli and *N. gonorrhoeae*, and to determine if hydrogen peroxide-producing lactobacilli can inhibit *N. gonorrhoeae in vivo*. As reviewed in the discussion section above, two main conclusions were made from our research. First, the *in vitro* mechanism utilized by *L. crispatus* and *L. jensenii* to inhibit *N. gonorrhoeae* was identified as the production of hydrogen peroxide. Second, the *in vivo* interaction between hydrogen-peroxide producing lactobacilli and *N. gonorrhoeae* may be more complicated than originally hypothesized. Future research may provide better understanding of this complicated interaction.

V. Future Studies. During the execution of this work, several ideas for future projects arose that might provide improved insight into the *in vitro* and *in vivo* interaction between human vaginal lactobacilli and *N. gonorrhoeae*. The development of a broth co-culture system might provide an easier method to quantify the inhibition of *N. gonorrhoeae* by hydrogen peroxide-producing *Lactobacillus spp*. For example, monitoring the growth of gonococci in spent media from lactobacillus broth cultures might provide a more quantifiable method to measure inhibition and allow for the purification of factors responsible for inhibition. Although a good suggestion in theory, this endeavor is challenged by the media requirements of both organisms. We have yet to find a mutually supportive medium for both lactobacilli and gonococci, however, all possibilities have not been exhausted.

A second question generated from this work, which might be addressed, is why *L crispatus* and *L. jensenii* possess the same acidification rate despite the observation that *L. crispatus* produces more lactate than *L. jensenii*. A simple *in vitro* experiment could be utilized to confirm that lactate is the primary source of *in vitro* acidification. Specifically, the pH of L-MRS broth could be measured following the addition of defined amounts of lactate. If lactate is the primary source of acidification during lactobacillus growth, one would predict that the pH of media containing exogenous lactate at a concentration equal to that measured in lactobacillus broth cultures would be similar.

Our *in vivo* studies raised many questions concerning lactobacillus colonization within the murine vaginal tract. A better understanding of the colonization mechanisms utilized by lactobacillus in the mouse vagina (i.e., receptor-mediated versus biofilm

formation) might help focus future studies on the potential reasons lactobacillus failed to inhibit *N. gonorrhoeae* during *in vivo* protection studies.

Finally, microarray analysis of both *L. crispatus* and *N. gonorrhoeae* could be utilized to identify changes in gene expression under varying conditions. Comparison of lactobacillus and gonococcal gene transcription when cultured *in vitro* or *in vivo* and in the absence or presence of each other might elucidate the reasons behind the results of our *in vivo* protection study results. This information might also provide vital information on gene induction *in vivo*, and clues as to the existence of interesting environmental stimuli in the host.

REFERENCES

1. Alcorn, T.M., H. Zheng, M.R. Gunther, D.J. Hassett, and M.S. Cohen. 1994. Variation in hydrogen peroxide sensitivity between different strains of *Neisseria gonorrhoeae* is dependent on factors in addition to catalase activity. Infection and Immunity 62:2138-2140.

2. Anathaswamy, H.N., and A. Eisenstark. 1977. Repair of hydrogen peroxide induced single-stranded breaks in *Escherichia coli* deoxyribonucleic acid. Journal of Bacteriology 130:187-191.

3. Antonio, M.A.D., S.E. Hawes, and S.L. Hillier. 1999. The identification of vaginal lactobacilli species and the demographic and microbiologic characteristics of women colonized by these species. Journal of Infectious Disease 180:1950-1956.

4. Aroutchera, A., D. Gariti, M. Simon, S. Shott, J. Faro, J.A. Somoes, A. Guguis, and S. Faro. 2001. Defense factors of vaginal lactobacilli. American Journal of Obstetrics and Gynecology 185:375-379.

5. Archibald, F.S., and M. Duong. 1981. Manganese and defense against oxygen toxicity in *Lactobacillus plantarum*. Journal of Bacteriology 154:442-451.

6. Archibald, F.S., and M. Duong. 1986. Superoxide dismutase and oxygen toxicity defense in the genus *Neisseria*. Infection and Immunity 51:631-641.

7. Barefoot, S.F. and T.R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Applied and Environmental Microbiology 45:1808-1815.

8. Barron, E.S.G., and A.B. Hastings. 1933. Studies on biological oxidations produced by gonococci. Journal of Biological Chemistry 97:691-715.

9. Bartlett, J.G., A.B. Onderdonk, and E. Drude. 1977. Quantitative bacteriology of the vaginal flora. Journal of Infectious Disease 136:271-277.

10. Bauer, G. 2001. Lactobacilli-mediated control of vaginal cancer through specific reactive oxygen species interaction. Medical Hypotheses 57:252-257.

11. Boris, S., J.E. Suarez, F. Vazquez, and C. Barbes. 1998. Adherence of human Vaginal Lactobacilli to Vaginal Epithelial Cells and Interaction with Uropathogens. Infection and Immunity 66:1985-1989.

12. Boskey, E.R., K.M. Telsch, K.J. Whaley, T.R. Moench, and R.A. Cone. 1999. Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Infection and Immunity 67:5170-5175.

13. Boskey, E.R., R.A. Cone, K.J. Whaley, and T.R. Moench. 2001. Origins of Vaginal Acidity: high D/L lactate ratio is consistent with bacteria being the primary source. Human Reproduction 16:1809-1813.

14. Braude, A.I., L.B. Corbeil, S. Levine, J. Ito, and J.A. McCutchan. 1978. Possible influence of cyclic menstrual changes on resistance to the gonococcus, p. 328-337. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunology of *Neisseria gonorrhoeae*. American Society of Microbiology Publishing, Washington, D.C.

15. Brooker, B.E., and R. Fuller. 1975. Adhesion of lactobacilli to chicken crop epithelium. Journal of Ultrastructure Research 52:21-31.

16. Brooks, G.F., J.S. Butel, and S.A Morse. 1998. Normal Microbial Flora of the Human Body, p. 177-181. *In* Jawetz, Melnick, and Adelberg's Medical Microbiology, 21st ed. Appleton and Lange Publishing, Stamford, CT.

17. Brown, W.J. 1982. Variations in the vaginal bacterial flora. Annals of Internal Medicine 96:931-934.

18. Bruce, A.W. 1973. Recurrent urethritis in women. Canadian Medical Association Journal 108:973-976.

19. Bruno, M.E.C., and T.J. Montville. 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 59:3003-3010.

20. Centers of Disease Control. 1999. Summary of notifiable diseases, United States, 1998. Morbidity and Mortality Weekly Report 47:1-93.

21. Chan, R.C.Y., A.W. Bruce, and G. Reid. 1984. Adherence of cervical, vaginal, and distal urethral normal microbial flora to human uroepithelial cells and inhibition of adherence of gram-negative uropathogens by competitive exclusion. Journal of Urology 131:596-601.

22. Chan, R.C.Y., G. Reid, R.T. Irvin, A.W. Bruce, and J.W. Costerton. 1985. Competitive exclusion of uropathogens from human uroepithelial cells by lactobacillus whole cells and cell wall fragments. Infection and Immunity 47:84-89.

23. Chen, C., and S.A. Morse. 1999. *Neisseria gonorrhoeae* bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. Microbiology 145:2967-2975.

24. Chow, A.W., and K.H. Bartlett. 1989. Sequential assessment of vaginal microflora in healthy women randomly assigned to tampon or napkin use. Reviews of Infectious Diseases 11(Sup 1):S68-S74.

25. Christensen, D.P., and R.W. Hutkins. 1992. Collapse of the proton motive force from *Listeria monocytogenes* caused by bactericidal produced by *Pediococcus acidilactici*. Applied and Environmental Microbiology 58:3312-3315.

26. Cohen, M.S., J.G. Cannon, A.E. Jerse, L.M. Charniga, and L.G. Whicker. 1994. Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. Journal of Infectious Disease 169:532-537.

27. Cohen, M.S., Y. Chai, B.E. Britigan, W. McKenna, J. Adams, T. Svendsen, K. Bean, D.J. Hassett, and P.F. Sparling. 1987. Role of extracellular iron in the action of quinone antibiotic streptonigrin: mechanisms of killing and resistance of *Neisseria gonorrhoeae*. Antimicrobial Agents Chemotherapeutics 31:1507-1513.

28. Cohen, M.S., J.R. Black, R.A. Proctor, and P.F. Sparling. 1984. Host defenses and the vaginal mucosa. A re-evaluation. Scandinavian Journal of Nephrology Supplement 86:13-22.

29. Conway, P.L., and S. Kjellberg. 1989. Protein-mediated adhesion of *Lactobacillus fermentum* strain 737 to mouse squamous epithelium. Journal of General Microbiology 135:1175-1186.

30. Cruicksank, R., and A. Sharman. 1934. The biology of the vagina in the human subject II. The bacterial flora and secretion of the vagina at various age periods and their relation to glycogen in the vaginal epithelium. Journal of Obstetrics and Gynaecology British Empire 41:190-226.

31. Dahiya, R.S. and M.L. Speck. 1968. Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. Journal of Dairy Science 51:1068-72.

32. Demple, B., and S. Linn. 1982. 5,6-Saturated thymine lesions in DNA: production by ultraviolet light or hydrogen peroxide. Nucleic Acids Res. 10:3781-3789.

33. Doderlein A. 1894. Die scheidensekretunterschugen. *Zentralbl Gynakol* 18:10-14.

34. DuPlessis, E.M., and L.M.T. Dicks. 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri, and L. johnsonii*. Current Microbiology 31:114-118.

35. Eschenbach, D.A., S.S. Thwin, D.L. Patton, T.M. Hooten, A.E. Stapleton, K. Agnew, C. Winter, A. Meir, and W.E. Stamm. 2000. Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. Clinical Infectious Diseases 30:901-907.

36. Eschenbach, D.A. 1989. Prevalence of Hydrogen Peroxide-Producing *Lactobacillus species* in normal women and women with bacterial vaginosis. Journal of Clinical Microbiology 27:251-256.

37. Eskow, R.N., and W.J. Loesche, 1971. Oxygen tensions in the human oral cavity. Achieves of Oral Biology 16:1127-1128.

38. Famularo, G., M. Pieluigi, R. Coccia, P. Mastroiacovo, and C. DeSimone. 2001. Microecology, bacterial vaginosis, and probiotics: perspectives for bacteriotherapy. Medical Hypotheses 56:421-430.

39. Fenchel, T. 2002. Microbial behavior in a heterogeneous world. Science 296:1068-1071.

40. Fontaine, E.A., and D. Taylor-Robinson. 1990. Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli. Journal of Applied Bacteriology 69:326-331.

41. Fu, K., D. Hassett, and M.S. Cohen. 1989. Oxidative stress in *Neisseria gonorrhoeae*: adaptation and effects on L-(+)-lactate dehydrogenase activity. Infection and Immunity 57:2173-2178.

42. Fuller, R. 1975. Nature of the Determinant responsible for the adhesion of lactobacilli to chicken crop epithelial cells. Journal of General Microbiology 87:245-250.

43. Galask, R.P., B. Larsen, and M.J. Ohm. 1976. Vaginal flora and its role in disease entities. Clinical Obstetrics and Gynecology 19:61-81.

44. Gilbert, P., T. Maira-Litran, A.J. McBride, A.H. Rickard, and F.W. Whyte. 2002. The physiology and collective recalcitrance of microbial biofilm communities. Advanced Microbial Physiology 46:202-256.

45. Ginkel, P.D., D.E. Soper, R.C. Brown, and H.P. Dalton. 1993. The vaginal flora in postmenopausal women: the effects of estrogen replacement. Infectious Diseases in Obstetric Gynecology 1:94-97.

46. Giorgi, A., S. Torriani, F. Dellaglio, G. Bo, E. Stola, and L. Bernuzzi. 1987. Identification of vaginal lactobacilli from asymptomatic women. Microbiologica 10:377-384.

47. Goplerud, C.P., M.J. Ohm, and R.P. Galask. 1976. Aerobic and anaerobic flora of the cervix during pregnancy and the puerperium. American Journal of Obstetrics and Gynecology 126:858-868.

48. Goodman, H.M. 1994. Basic medical endocrinology. Second Edition. p. 285-290. Raven Press: New York, N.Y.

49. Gorbach, S.L., K.B Menda, H. Thadepalli, and L. Keith. 1973. Anaerobic microflora of the cervix in healthy women. American Journal of Obstetrics and Gynecology 117:1053-1055.

50. Gupta, K., Stapleton, A.E., Hooten, T.M., Roberts, P.L., Fennell, C.L., and W.E. Stamm. 1998. Inverse association of hydrogen peroxide-producing lactobacilli and vaginal *Escherichia coli* colonization in women with recurrent urinary tract infections. Journal of Infectious Disease 178:446-50.

51. Haas, A., and W. Goebel. 1992. Microbial strategies to prevent oxygen-dependent killing of phagocytes. Free Radical Res. Communications 16:137-157.

52. Handsfield, H.H. 1990. *Neisseria gonorrhoeae* in principles and practice of infectious disease, p. 1613-1631. eds. J.E. Bennet, R.G. Douglas, and G.L. Mandell. 3rd ed. Churchill Livingstone Publishers, New York, NY.

53. Harris, J.W., and J.H. Brown. 1928. The bacterial content of the vagina and uterus on the fifth day of the normal puerperium. Bulletin of John Hopkins Hospital 43:190-200.

54. Harty D.W., H.J. Oakey, M. Patrikakis, E.B. Hume, and K.W. Knox. 1994. Pathogenic potential of lactobacilli. International Journal of Food Microbiology 24:179-89.

55. Havenaar, R., B. Ten Brink, and J.H.J. Huis In't Veld. 1992. Selection of strains for probiotic use, p. 209-224. In R. Fuller (ed), Probiotics, the scientific basis. Chapman and Hall Publishers, London.

56. Hawes, S.E., S.L. Hillier, J. Benedetti, C.E. Stevens, L.A., P. Wolner-Hanssen, and K.K. Holmes. 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. Journal of Infectious Disease 174:1058-1063.

57. Hendriksson, A., R. Szewzyk, and P.L. Conway. 1991. Characteristics of the adhesive determinants of *Lactobacillus fermentum* 104. Applied and Environmental Microbiology 57:499-502.

58. Hill, G.B., K.K. St. Claire, and L.T. Gutman. 1995. Anaerobes predominate among the vaginal microflora of prepubertal girls. Clinical Infectious Disease 20(Sup 2):S269-270.

59. Hillier, S.L., 1999. Normal vaginal flora, p. 191-205. *In* K.K. Holmes, P.F. Sparling, P. Mardh, S.M. Lemon, P. Piot, and J.N. Nasserheit, Sexually Transmitted Disease. McGraw-Hill Publishing, New York, NY.

60. Hillier, S.L., and R.J. Lau. 1997. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. Clinical Infectious Disease 25:S123-S126.

61. Hillier, S.L., M.A. Krohn, S.J. Klebanoff, and D.A. Eschenbach. 1992. The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. Obstetrics and Gynecology 79:369-373.

62. Hillier, S.L. M.A. Krohn, R.P. Nugent, and R.S. Gibbs. 1992. Characteristics of three vaginal flora patterns assessed by Gram stain among pregnant women. American Journal of Obstetrics and Gynecology 166:938-944.

63. Hite, K.E., H.C. Hesseltine, L. Goldstein. 1947. A study of the bacterial flora of the normal and pathologic vagina and uterus. American Journal of Obstetrics and Gynecology 53:233-240.

64. Hughes, V.L. and S.L. Hillier. 1990. Microbiologic characteristics of lactobacillus products used for colonization of the vagina. Obstetrics and Gynecology 75:244-248.

65. Isolauri, E., M. Juntunen, T. Rautanen, P. Sillanaukee, and T. Koivula. 1991. A human Lactobacillus strain (*Lactobacillus casei* sp strain GG) promotes recovery from acute diarrhea in children. Pediatrics 88:90-97.

66. Jack, R.W., J.R. Tagg, and B. Ray. 1995. Bacteriocins of Gram positive bacteria. Microbiological Review 59:171-200.

67. Jacoby, R.O., and J.G. Fox. 1984. Biology and disease of mice. p. 41-44. *In* Laboratory Animal Medicine, J.G. Fox, B.J. Cohen, and F.M. Loew (ed.). Academic Press, Inc. New York, NY.

68. Jerse, A.E., M.S. Cohen, P.M. Drown, L.G. Whicker, S.F. Isbey, H.S. Seifert, and J.G. Cannon. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in males. Journal of Experimental Medicine 179:911-920.

69. Jerse, A.E. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. Infection and Immunity 67:5699-5708.

70. Jerse A.E, E.T. Crow, A.N. Bordner, I. Rahman, C.N. Cornelissen, T.R. Moench, K. Mehrazar. 2002. Growth of *Neisseria gonorrhoeae* in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. Infection and Immunity 70:2549-58

71. Johnson, J.L., C.F. Phelps, C.S. Cummins, J. London, and F. Gasser. 1980. Taxonomy of the *Lactobacillus acidophilus* group. International Journal of Systematic Bacteriology 30:53-68.

72. Johnson, S.R., C.R. Petzold, and R.P. Galask. 1985. Qualitative and quantitative changes in the vaginal microbial flora during the menstrual cycle. American Journal of Reproductive Immunology 9:1-5.

73. Johnson, S.R., B.M. Steiner, D.D. Cruce, G.H. Perkins, and R.J. Arko. 1993. Characterization of a catalase-deficient strain of *Neisseria gonorrhoeae*: evidence for the significance of catalase in the biology of *N. gonorrhoeae*. Infection and Immunity 61:1232-1238.

74. Kanatani, K., M. Oshimura, K. Sano. 1995. Isolation and characterization of acidocin A and cloning the bacteriocin gene from *Lactobacillus acidophilus*. Applied and Environmental Microbiology 61:1061-1067.

75. Kaye, D., and M.E. Levison. 1977. In vitro inhibition of growth of *Neisseria gonorrhoeae* by genital microorganisms. Sexual Transmitted Diseases 4:1-3.

76. Knapp, J.S., and V.L. Clark. 1984. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. Infection and Immunity 46:176-181.

77. Kraus, S.J., and N. Ellison. 1974. Resistance to gonorrhea possibly mediated by bacterial interference. Applied Microbiology 27:1014-1016.

78. Larsen, B. 1993. Vaginal flora in health and disease. Clinical Obstetrics and Gynecology 36:107-121.

79. Larsen, B., and R.P. Galask. 1982. Vaginal microbial flora: composition and influences of host physiology. Annals of Internal Medicine 96:926-930.

80. Larsen, B., and R.P. Galask. 1980. Vaginal Microbial Flora: practical and theoretic relevance. Obstetrics and Gynecology 55:100S-112S.

81. Larsen, B., and A.J. Markovetz. 1977. Role of estrogen in controlling the genital microflora of female rats. Applied and Environmental Microbiology 35:534-540.

82. Lengeler, J.W., G. Drews, and H.G. Schlegel (eds). 1999. Biology of the prokaryotes. p 263-266. Thieme Stuttgart Publishing: New York, NY.

83. Leroy, F., and L. de Vuyst. 2001. Growth of bacteriocin-producing *Lactobacillus sakei* strain CTC 494 in MRS broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for the growth of lactic acid bacteria. Applied and Environmental Microbiology 67:4407-4413.

84. Levison, M.E., L.C. Corman, E.R. Carrington, and D. Kaye. 1977. Quantitative microflora of the vagina. American Journal of Obstetrics and Gynecology 127:80-85.

85. Lin, J.K., and C.C. Lai. 1982. Effects of lactobacillus, antacids, and antibiotics on the levels of nitrite in the gastro-intestinal tracts of rats fed sodium nitrate. Food and Chemical Toxicology 20:197-204.

86. Mandar, R., and M. Mikelsar. 1996. Transmission of the mother's microflora to the newborn at birth. Biology of the Neonate 69:30-35.

87. Martin, H.L., B.A. Richardson, P.M. Nyange, L. Lavreys, S.L. Hillier, B. Chohan, K. Mandaliya, J.O. Ndinya-Achola, J. Bwayo, and J. Kreiss. 1999. Vaginal Lactobacilli, microbial flora, and risk of human immunodeficiency virus type I and sexually transmitted disease acquisition. Journal of Infectious Disease 180:1683-1688.

88. Martius, J., M.A. Krohn, S.L. Hillier, W.E. Stramm, K.K. Holmes, D.A. Eschenbach. 1988. Relationship of vaginal *Lactobacillus species*, cervical *Chlamydia trachomatis*, and bacterial vaginosis to preterm birth. Obstetrics and Gynecology 71:89-95.

89. McBride, M.E., W.C. Duncan, and J.M. Knox. 1978. Method for studying the role of indigenous cervical flora in colonization by *Neisseria gonorrhoeae*. British Journal of Venereal Disease 54:386-393.

90. McGroarty, J.A., L. Tomeczek, D.G. Pond, G. Reid, and A.W. Bruce. 1992. Hydrogen peroxide production by *Lactobacillus species*: correlation with susceptibility to the spermicidal compound nonoxynol-9. Journal of Infectious Disease 165:1142-1144.

91. McLean, N.W., and I.J. Rosenstein. 2000. Characterization and selection of a Lactobacillus species to re-colonize the vagina of women with recurrent vaginosis. Journal of Medical Microbiology 49:543-552.

92. Mestecky, J., and M.W. Russell. 2001. Induction of mucosal immune responses in the human genital tract. FEMS Immunology and Medical Microbiology 27:351-355.

93. Moat, A.G., and J.W. Foster. 1995. Microbial Physiology. 3rd ed. p. 367-376. Wiley-Liss Publishing:New York, NY.

94. Moore, T.D., and P.F. Sparling. 1995. Isolation and identification of glutathione peroxidase homolog gene, *gpxA* present in *Neisseria meningitides* but absent in *N. gonorrhoeae*. Infection and Immunity 63:1603-1607.

95. Morgan, R.W., M.F. Christman, G. Jacobson, G. Storz, and B.N. Ames. 1986. Hydrogen peroxide inducible proteins in *Salmonella typhimurium* overlap with heat shock and stress proteins. Proceedings of National Academy of Science-USA 83:8059-8063.

96. Neidhardt, F.C., J.L. Ingraham, and M. Schaechter (eds). 1990. Physiology of the bacterial cell: a molecular approach. p. 160-166. Sinauer Associates Incorporated Publishing, New York, NY.

97. Norrod, P., and S.A. Morse. 1979. Absence of superoxide dismutatase in some strains of *N. gonorrohoeae*. Biochemical Biophysiology Res. Communications. 90:1287-1294.

98. Ocana, V.S., A.A. Pesce de Ruiz Holgado, and M.E. Nader-Macias. 1999. Characterization of a bacteriocin-like substance produced by vaginal *Lactobacillus salivarius* strain. Applied and Environmental Microbiology 65(12):5631-5635.

99. Ocana, V.S., A.A. Pesce de Ruiz Holgado, and M.E. Nader-Macias. 1999a. Selection of vaginal H_2O_2-generating *Lactobacillus species* for probiotic use. Current Microbiology 38:279-284.

100. Ocana, V.S., A.A. Pesce de Ruiz Holgado, and M.E. Nader-Macias. 2001. Growth inhibition of *Staphylococcus aureus* by hydrogen peroxide-producing *Lactobacillus paracasei* subspecies *paracasei* isolated from the human vagina. FEMS Immunology and Medical Microbiology 23:87-92.

101. Ogawa, M., K. Shimizu, K. Nomoto, R. Tanaka, T. Hamabata, S. Yamasaki, T. Takeda, and Y. Takeda. Inhibition of in vitro growth of shiga toxin-producing *Escherichia coli* O157:H7 by probiotic lactobacillus strains due to production of lactic acid. International Journal of Food Microbiology 68:135-140.

102. Olm, M.J., and R.P. Galask. 1975. Bacterial flora of the cervix from 100 prehysterectomy patients. American Journal of Obstetrics and Gynecology 122:683-687.

103. Onderdonk, A.B., G.R. Zamarchi, J.A. Walsh, R.D. Mellor, A. Munoz, and E.H. Kass. 1986. Methods for quantitative and qualitative evaluation of vaginal microflora during menstruation. Applied and Environmental Microbiology 51:222-229.

104. Paraje, M.G., I. Albesa, and A.J. Eraso. 2000. Conservation in probiotic preparations of lactobacillus with inhibitory capacity on other species. New Microbiology 23:423-431.

105. Parent, D., M. Bossens, D. Bayot, C. Kirkpatrick, F. Graf, F.E. Wilkinson, and R.R. Kaiser. 1996. Therapy of bacterial vaginosis using exogenously-applied *Lactobacilli acidophili* and a low dose of estriol: a placebo-controlled multicentric clinical trial. Arzneimittelforschung 46:68-73.

106. Parham, P. 2000. The immune system, p. 6-30. Garland Publishing, New York, NY.

107. Piccolomini, R., C. Di Ilio, A. Aceto, A. Faraone, L. Cellini, G. Ravagnan, and G. Federici. 1989. Glutathione transferase in bacteria: subunit composition and antigenic characterization. Journal of General Microbiology 135:3119-3125.

108. Puapermpoonsiri, S., N. Kato, K. Watanabe, K. Ueno, C. Chongsomchai, and P. Lumbiganon. 1996. Vaginal microflora associated with bacterial vaginosis in Japanese and Thai pregnant women. Clinical Infectious Diseases 23:748-752.

109. Pybus, V., and A.B. Onderdonk. 1999. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. Microbes and Infection 1:285-292.

110. Rautio, M., H. Jousimies, and H. Kauma. 1999. Liver abscess due to *Lactobacillus rhamnosus* strain indistinguishable from *L. rhamonsus* strain GC. Clinical Infectious Diseases 28:1159-1160.

111. Redondo-Lopez V., R.L. Cook, and J.D. Sobel. 1990. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Review of Infectious Diseases 12:856-72.

112. Reid, G., A.L. Servin, A.W. Bruce, and H.J. Busscher. 1993. Adhesion of three lactobacillus strains to human urinary and intestinal epithelial cells. Microbios. 75:57-65.

113. Reid, G., and A.W Bruce. 2001. Selection of Lactobacillus for urogenital probiotic applications. Journal of Infectious Diseases 183(Sup1):S77-S81

114. Reid, G., J.A. McGroarty, P.A. Gil Domingue, A.W. Chow, A.W. Bruce, A. Eisen, and J.W. Costerton. 1990. Coaggregation of urogenital bacterial *in vitro* and *in vivo*. Current Microbiology 20:47-52.

115. Reid, G., J.A. McGroarty, R. Angotti, and R.L. Cook. 1988. Lactobacillus inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Canadian Journal of Microbiology 34:344-351.

116. Reid, G. and J.D. Sobel. 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Review of Infectious Disease 9:470-487.

117. Rogosa, M. and M.E. Sharpe. 1960. Species differentiation of human vaginal lactobacilli. Journal of General Microbiology 23:197-201.

118. Saigh, J.H., C.C. Sanders, and W.E. Sanders, Jr. 1978. Inhibition of *Neisseria gonorrhoeae* by aerobic and facultatively anaerobic components of the endocervical flora: evidence for a protective effect against infection. Infection and Immunity 19:704-710.

119. Sautter, R.L., and W.J. Brown. 1980. Sequential vaginal cultures from normal young women. Journal of Clinical Microbiology 11:479-484.

120. Schwebke, J.R., C.M. Richey, and H.L. Weiss. 1999. Correlation of behaviors with microbiological changes in vaginal flora. Journal of Infectious Disease 180:1632-1636.

121. Shtibel, R. 1976. Inhibition of growth of *Neisseria gonorrhoeae* by bacterial interference. Canadian Journal of Microbiology 22:1430-1436.

122. Smith, H., E.A. Yates, J.A. Cole, and N.J. Parsons. 2001. Lactate stimulation of gonococcal metabolism bin media containing glucose: mechanism, impact on pathogenicity, and wider implications of other pathogens. Infection and Immunity 69:6565-6572.

123. Snell, G.D. 1941. Reproduction, p. 55-58. *In* Biology of the laboratory mouse, G.D. Snell (ed), Dover Publications, Inc. New York, NY.

124. Solar-Garcia, A. 2002. Ph.D. dissertation. Uniformed Services University. Bethesda, MD.

125. Song, Y.L., N. Kato, C.X. Liu, Y. Matsumiya, H. Kato, and K. Watanabe. 2000. Rapid Identification of 11 human intestinal *Lactobacillus species* by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiology Letters 187:167-173.

126. Sookkhee, S., M. Churlasiri, and W. Prachyabrued. 2001. Lactic acid bacteria from health oral cavity of Thai volunteers: inhibition of oral pathogens. Journal of Applied Microbiology 90:172-179.

127. St. Amant, D., and A.E. Jerse. Personal communication.

128. Stainer,, R.Y., E.A. Adelberg, and J, Ingraham. 1976. Gram-positive bacteria: the actinomycete line, p. 678-684, *In* The Microbial World, 4th Ed. Prentice-Hall Publishing, Inc., Engelwood Cliffs, NJ.

129. Steward-Tull, D.E.S. 1964. Evidence that vaginal lactobacilli do not ferment glycogen. American Journal of Obstetrics and Gynecology 88:676-679.

130. Sutherland, I.W. 2001. The biofilm matrix-an immobilized but dynamic microbial environment. Trends in Microbiology 9:222-227.

131. Tannock, G.W., Munro, K. Harmsen, H.J., Welling, G.W., and P.K. Smar. 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Applied and Environmental Microbiology 66:2578-2588.

132. Thompson, R. and A. Johnson. 1950. Inhibitory action of saliva on the diphtheria bacillus: hydrogen peroxide, the inhibitory agent produced by salivary streptococci. Journal of Infectious Disease 88:81-85.

133. Tramer, J. 1966. Inhibitory effect of *Lactobacillus acidophilus*. Nature 211:204-205.

134. Tseng, H., Y. Srikhanta, A.G. McEwan, and M.P. Jennings. 2001. Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. Molecular Microbiology 40:1175-1186.

135. Vallor, A.C., M.A.D. Antonio, S.E. Hawes, and S.L. Hillier. 2001. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. Journal of Infectious Disease 184:1431-1436.

136. van der Vegt, W., H.C. van der Mei, J. Noordmans, and H.J. Busscher. 1991. Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. Applied and Environmental Microbiology 35:766-770.

137. van der Wielen, P.W., L.J. Lipman, F. van Knapen, and S. Biesterreld. 2002. Competitive exclusion of *Salmonella enterica* serovar Enteritidis by *Lactobacillus crispatus* and *Closteridium lactatifermentans* in sequencing fed batch culture. Applied and Environmental Microbiology 68:555-559.

138. Velraeds, M.M., B. van de Belt-Gritter, H.C. van der Mei, G. Reid, and H.J. Busscher. 1998. Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. Journal of Medical Microbiology 47:1081-5.

139. Velraeds, M.M.C., H.C. van der mei, G. Reid, and H.J. Busscher. 1996. Inhibition of Initial Adhesion of Uropathogenic *Enterococcus faecalis* by Biosurfactants from *Lactobacillus* isolates. Applied and Environmental Microbiology 62:1958-1963.

140. Ventura, M., I.A. Casa, L. Morelli, and M.L. Callegari. 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus sp.* isolated from fecal and vaginal samples. Systematic and Applied Microbiology 23:504-509.

141. Vincent, J.G., R.C. Veomett, and R.F. Riley. 1959. Antibacterial activity associated with *Lactobacillus acidophilus*. Journal of Bacteriology 78:477-484.

142. Wagner, G., R.J. Levin, and L. Bohr. 1988. Diaphragm insertion increases human vaginal oxygen tension. American Journal of Obstetrics and Gynecology 158:1040-1043.

143. Wagner, G., L. Bohr, P. Wagner, and L.N. Petersen. 1984. Tampon-induced changes in oxygen and carbon dioxide tensions. American Journal of Obstetrics and Gynecology 148:147-150.

144. Wagner, G., and R.J. Levin. 1978. Oxygen tension of the vaginal surface during sexual stimulation in the human. Fertility and Sterility 30:50.

145. Weinstein, L., and J.H. Howard. 1939. The effect of estrogenic hormone on the H-ion concentration and the bacterial content of the human vagina with special reference to the Doderlein bacillus. American Journal of Obstetrics and Gynecology 37:698-703.

146. Weinstein, L. 1938. The bacterial flora of the human vagina. Yale Journal of Biological Medicine 10:247-260.

147. Weiss, S.J. 1986. Oxygen, iscemia, and inflammation. Acta Physiol. Scand. Sup. 548:9-37.

148. Wheater, D.M., A. Hirsch, A.T.R. Mattick. 1952. Possible 'identity of lactobacillin' with hydrogen peroxide produced by lactobacilli. Nature 170: 623-624.

149. Wilks, M., and S. Tabaqchali. 1987. Quantitative bacteriology of the vaginal flora during the menstrual cycle. Journal of Medical Microbiology 24:241-245.

150. Whittenbury, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. Journal of General Microbiology 35:13-26.

151. Wylie, J.G., and A. Henderson. 1969. Identity and glycogen-fermenting ability of lactobacilli isolated from the vagina of pregnant women. Journal of Medical Microbiology 2:363-366

152. Zheng, H., D.J. Hassett, K. Bean, and M.S. Cohen. 1992. Regulation of catalase in *Neissera gonorrhoeae*: effects of oxidant stress and exposure to human neutrophils. Journal of Clinical Investigation 90:1000-1006.

153. Zheng, H., T.M. Alcorn, and M.S. Cohen. 1994. Effects of hydrogen peroxide-producing lactobacilli on *Neissera gonorrhoeae* growth and catalase activity. Journal of Infectious Disease 170:1209-1215.